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Reliable intermediate biological markers for breast cancer risk, than can be easily detected in both pre- and post- menopausal women, do not exist at the present time. For more than 20 years, the ability to access breast ductal fluid through the nipple has prompted initiatives to develop a PAP-like test for breast cancer. Yields were variable, not every woman yielded fluid, and there was no assurance of obtaining samples from the entire length of the ducts. In this proposal, we will use a 1) a facile ductal lavage (DL) technique using cannulating catheters which flushes each duct to yield thousands of ductal cells. 2) a panel of markers consisting of three genes, Cyclin D2, Twist and retinoic acid receptor •2 (RAR•2), which are aberrantly hypermethylated in breast cancer cells. We will standardize the techniques using fluid from cancer patients, and then evaluate the frequency of cells positive by MSP assays in ductal lavage obtained from women with a high risk of developing breast cancer, such as patients with lobular carcinoma, patients with cancer in one breast, and those with mammographically suspicious lesions. Thus, we aim to develop a PAP test for the breast.

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Introduction:

Detected early, breast cancer is an eminently curable disease. However, reliable intermediate biological markers for breast cancer risk, that can be easily detected in both pre- and post-menopausal women, do not exist at the present time. Conventional cytological examination of breast cells is a fairly reliable test to detect breast cancer cells. However, its sensitivity and specificity could be complemented and supplemented with molecular diagnostic tests. It is increasingly clear that silencing of gene expression by promoter hypermethylation is a common feature of cancer and is seldom seen in normal tissues except for imprinted genes and genes on the inactive X chromosome. Using a sensitive assay called methylation specific PCR (1), initial studies show that MSP can detect 1 methylated gene copy in 1000 unmethylated gene copies, attesting to the sensitivity of this approach. Importantly, the assays are highly specific in that no abnormal methylation was detected in serum DNA if the same alteration was not present in the primary tumor. Promoter methylation has been reported in 15-50% of primary breast tumors for the following genes: 14.3.3 sigma (2,3), RAR-beta (4, 5), cyclin D2 (6), HOXA5 (7), Twist (8), RASSF1A (9, 10, 11), HIN-1 (12) and NES-1 (13), to name a few.

Body:

Month 1 to 6: Optimize conditions for duplex or multiplex assays for the 5 markers RARβ, RASSF1A, Twist, cyclin D2, and HIN-1 using fluid spiked with varying numbers of tumor cells.

We standardized quantitative methylation specific PCR (14) for all five genes. Here the readout is of the percentage of methylation present in each sample, and one can calculate how many microgram equivalents of methylated DNA are present in the particular sample. This method is able to detect down to 20 picogram of methylated DNA in vast excess of unmethylated DNA from normal cells. However, DNA recovered from these samples is a limiting factor, which we have now addressed by developing a multiplex MSP assay.

Then test the MSP markers on ductal lavage from tumor-containing breast of 25 women just prior to surgery for known lesion.

Ductal lavage has been completed on 25 women in the WIRB approved trial on ductal lavage, just prior to surgery for a biopsy proven lesion. These fluids have now undergone cytology analysis. Once the analysis is complete, DNA will be extracted from the cytospin preparations and tested for the presence of methylated genes.

Months 7 to12: If sample is limiting, perform RARβ, RASSF1A, Twist, cyclin D2, and then HIN-1 in a stepwise fashion, going from the highest (85%) to the lowest (30%) incidence markers. Samples may need pre-amplification to enable use of all 5 markers on the varying numbers of tumor cells obtained by ductal lavage. Standardize this methodology. Then test the MSP markers on ductal lavage from tumor-containing breast of 25 additional women just prior to surgery for known lesion.

We have made considerable progress on this specific aim. Knowing that cells from ductal lavage will always remain a limiting factor, we have developed a multiplex quantitative Methylation specific PCR. This PCR test allows us to test all 5 genes with little limitation of sample size. It is important to ascertain whether the increase in sensitivity of this method does not result in loss of specificity. Also, we would like to set the normal range of values from normal tissue, thus setting the cut-off value above which the values can be considered abnormal. This has been completed for each gene using reduction mammoplasty specimens. Also to determine if epithelial or stromal cells provide false positive values for some genes, we microdissected cells from sections of benign and normal breast sections and performed the same assay. The cut-off values have been set at the higher limit of 1% methylation as being normal values.

We plan to use a novel quantitative multiplex methylation specific PCR (MQ-MSP) assay, recently developed by us, on cells from the fixed and stained cytospin preparation to detect 5 genes on DNA obtained from the ductal cells of breast cancer patients. The five genes, Cyclin D2, RAR-β, Twist, RASSF1A, and HIN-1, were selected because they are specifically and frequently (more than 30%) hypermethylated in breast tumor cells, but are not methylated in normal blood cells or normal breast cells. Actin is the 6th gene which is amplified as an internal control for normalization of the reactions based on total DNA content. Multiplex Q-MSP test is very specific, is conducted in a multi-well format suited to large-scale analysis, and provides finite values for the amount of methylated DNA present in each sample as the reaction is progressing in real time (our unpublished data). More importantly, the test is objective, and its sensitivity is well established. The panel of these 5 markers can detect breast cancer (as early as DCIS) in 100% of women (our unpublished data). Most tumors contain 1 or more markers hypermethylated. Combining the sensitivity and objectivity of the test with the specificity of the markers, we have a good chance of identifying women at high risk of developing breast cancer.

Standardization of the Multiplex Q-MSP.

Quantitative real-time -MSP (Q-MSP) test is very specific, is conducted in a multi-well format suited to large-scale analysis, and provides values for the relative amount of methylated and unmethylated DNA present in each sample as the reaction is progressing (in real time). More importantly, unlike conventional MSP (1), the test is objective, and its sensitivity is well established.

We have standardized this assay in a multiplexed format for 6 genes. Using only one aliquot (out of 10 ul) of the sodium bisulfite treated DNA, in Step 1 all the genes in the panel are amplified in one tube. The first reaction contained 6 gene-specific "external" primers that were designed to anneal to genomic DNA independent of methylation status of the genome (do not contain any CpGs in their own sequence). In step 2, this DNA is diluted, and subjected to Q-MSP for both methylated and unmethylated sequences, using 3 gene-specific primers (2 flanking primers and 1 probe) for the CpG containing region. We have standardized the analysis for the RASSF1A gene in Step 2. We determined that human sperm DNA (HSD) is unmethylated (100%), and that MDA MB 231 breast cancer cell line DNA is methylated (100%) at the RASSF1A promoter (Figure 1A). Human sperm DNA and 231 DNA was mixed in roughly equal proportions. 20 ng of the mixture

DNA was amplified by 35 cycles in a 25 microliter PCR reaction using forward and reverse primers for RASSF1A, RARβ, Cyclin D2, Twist, Hin-1, and actin (6 pairs of primers used simultaneously) in the multiplex PCR reaction. This DNA was then used as a source of the "standard".

To provide a quantitative estimate of the amount of methylation present in each sample, the following was done. In each run, the multiplexed DNA "standard" was serially diluted and amplified with RASSF1A primers specific for either methylated or unmethylated DNA. By plotting Ct vs dilution, a linear trend line with a correlation coefficient >.99 was established. It is also established that the slope of the U curve and M curve are approximately the same, indicating both reactions are occurring with the same efficiency (e.g. 2 fold increase per cycle, 10 fold increase every 3.3 cycles). DNA dilution equivalents in the test samples were extrapolated off the standard curve for 20 ng HSD and 231 genomic DNA. % Methylated DNA is calculated by-.

% Methylated DNA= [M/(U+M)]100

Figure 1A

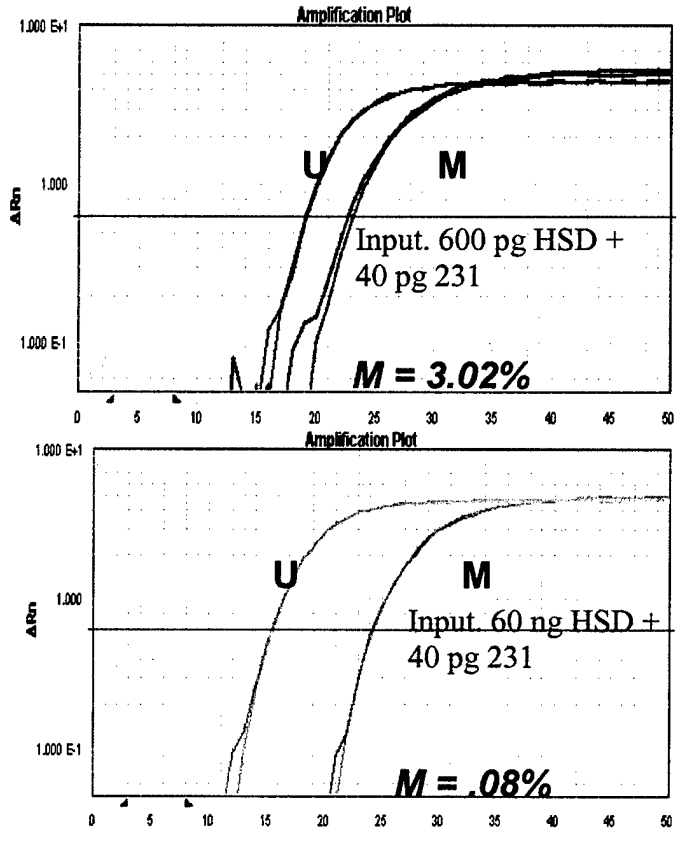

Figure 1B

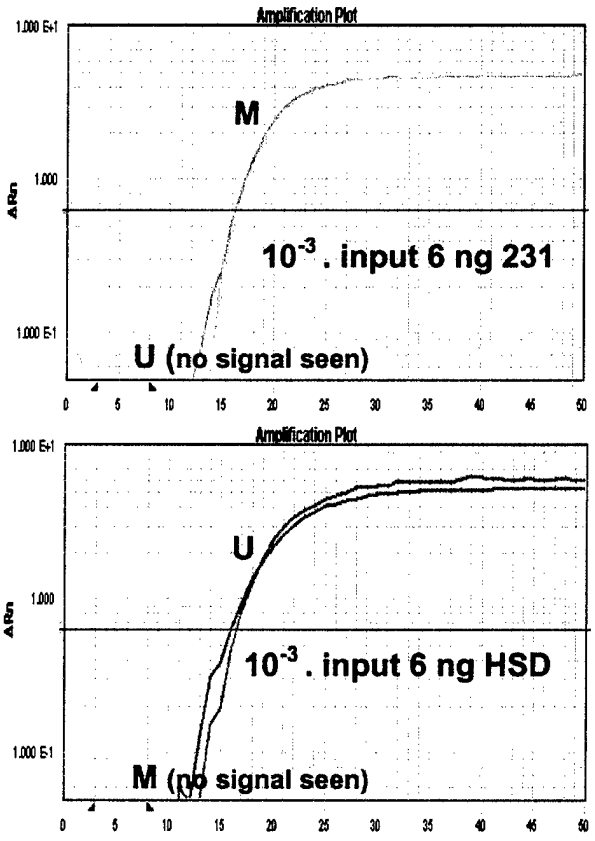

Figure 1A. M-Q-MSP is sensitive. Panel A: Multiplexed DNA (mixture) was diluted (10-3) and Q-MSP was performed using primers specific for the unmethylated (U) or methylated (M) RASSF1A promoter. Panel A: 600 pg of HSD plus 40 pg 231DNA. Panel B: 60 ng of HSD with 40 pg of 231 DNA.

Figure 1B. M-Q-MSP is specific. Primers for methylated DNA (231) do not detect unmethylated HSD (panel A), and vice versa (panel B). One ul of the 1 in 1000 dilution of the multiplex reaction was used in the Q-MSP reaction.

Table 1

RASSF1A Methylation in Ductal Carcinoma				
ID		U	M	% M
1	DCIS/ 1	81303.46	0.00	0.00
2	DCIS /1	186121.62	0.00	0.00
3	DCIS /1	7840.00	1661.54	17.49
4	DCIS /2	1221993.9	194693.08	14.00
5	DCIS/ 3	414613.88	46676.41	10.12
6	DCIS /3	3072.88	2803.76	47.71
7	DCIS /3	462237.84	121006.98	20.75
8	DCIS /3	511980.94	130374.00	20.30
9	IDC	60212.73	31235.53	34.16
10	IDC	3199.05	3121.15	49.38
11	IDC	86387.04	288.61	0.33
12	IDC	66219.05	0.00	0.00

Table 2

RASSF1A Methylation In Reduction Mammoplasty Specimens				
ID	U	M	% M	Age over 49
1	96731.95	0.00	0.00	
2	180330.70	0.00	0.00	X
3	155594.48	0.00	0.00	X
4	13343.36	0.00	0.00	
5	1792.47	0.00	0.00	
6	2103863.80	612793.20	18.00	X
7	42132.44	0.00	0.00	
8	255753.55	0.00	0.00	X
9	301421.44	0.00	0.00	X
10	9011.11	0.00	0.00	
11	342109.30	0.00	0.00	
12	185307.84	429523.60	69.00	
13	1580726.90	0.00	0.00	X
14	189218.97	0.00	0.00	X
15	9175290.00	0.00	0.00	
16	238118.89	0.00	0.00	
17	344951.38	0.77	0.00	
18	7317194.50	0.00	0.00	
19	8428731.00	0.00	0.00	
20	3189157.20	0.56	0.00	
21	1493306.80	0.00	0.00	
22	937980.30	0.00	0.00	
23	448169.25	3235.87	0.72	
24	32345.32	0.00	0.00	
25	2491771.50	0.00	0.00	
26	511569.62	0.00	0.00	
27	1032461.94	0.00	0.00	X

Table 1. By M-Q-MSP RASSF1A is frequently methylated in DCIS and invasive ductal carcinoma. Levels of RASSF1A methylation of ductal carcinoma in situ (Grade1, 2 or 3), and invasive carcinomas.

Table 2. Twenty-seven mammoplasty specimens of women with benign disease, including 19 samples of tissues from under the age of 50, and 8 samples from over the age of 50 were analyzed by M-Q-MSP. Sample#16 and Sample #12 contained 18%, and 69% values for methylated DNA levels. We are investigating the hyperplasias in these tissues more closely. Sample #23 contained less than 1% methylated alleles. No age-related methylation was observed in this small sampling.

Analysis of 28 specimens of normal peripheral blood cells showed abundant unmethylated DNA but less than 0.01% of methylated DNA. We then investigated the status of RASSF1A methylation in reduction mammoplasty specimens (Table 2).

Sample preparation and M-Q-MSP analysis. DNA will be extracted from the cytospin preparation following diagnosis and/or immunohistochemistry. A minimum of 20 breast

epithelial cells from either source is sufficient for MSP analysis. The coverslip is removed after soaking in xylene, the section is scraped and placed in 50-100 ul of TNES with proteinase K for digestion overnight. After centrifugation at 14K, the supernatant DNA is treated with sodium bisulfite, and extracted in 10 ul of water (1). One ul of this is adequate for performing multiplex and Q-MSP for 6 or more genes (our unpublished work). Amplicon sizes for the seven genes vary from 70-80 bp, and all of them will be PCR-amplified under identical experimental conditions.

The contents of a typical 96 well plate consists of the following: 1) 30 patient samples, two wells per sample (one well with U or and one well with M primers), 2) two standard DNA dilution curves (dilutions of 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9} in duplicate each for U and for M primer), two genomic DNA standards of known amounts of DNA (20 ng human sperm DNA and MDA MB 231 DNA determined by OD_{260}) in duplicate wells, 3) two multiplex DNA standards of known % M (200/13 copies and 20,000/13 copies U/M), and water controls in duplicate from the first PCR reaction that serve as No Template Control (NTC) for each primer set U and M.

Potential Problems.

Amount of DNA available may be too low: Small quantities of DNA will be extracted from ductal lavage cells of women with DCIS and small cancers, and even less from normal individuals. We have previously successfully conducted studies on ductal cells obtained from 150 women with and without cancer. In many instances, the pathologist deemed the sample not suitable for cytology- yet we were able to obtain results by conventional MSP. To enable us to use more markers and to circumvent this problem, we have now developed the multiplex approach- with the potential to analyze 100s of genes. The quantitative feature of this assay has allowed us to set the cutoff point for normal tissue at less than 1% methylation compared to the unmethylated signal.

Sensitivity may be too low: We have successfully multiplexed 6 gene sets, and shown data for RASSF1A (Figure 1, Table 1) that look very promising. The sensitivity of our methodology is at its desired level- we are able to detect down to 40 pg of hypermethylated DNA in presence of 40 ng unmethylated DNA. Improvements by changing magnesium concentration, primer locations, and other experimental PCR conditions will be tested. We would like our marker panel to distinguish between benign and atypical hyperplasia- this experiment has never been done.

Method may detect too many positives- may not be specific: In this situation, cytology analysis will be negative and Q-MSP will be positive for the same sample. We have conducted studies on 27 mammoplasty specimens, many of which had fibrocystic changes and a few had ductal hyperplasia. Nearly all of them were negative for methylation. Thus we do not expect rampant positivity in benign disease breasts. The two positive samples appear to be those with hyperplasias, and in one case, papilloma in the contralateral breast. The information obtained from this proposal will be regarded as hypothesis generating, providing the foundation for planning a more definitive clinical trial. In particular, this information will provide the statistics necessary for appropriate power calculation to design a definitive clinical trial.

Months 12-18: Test fluid from contralateral ducts (tumor-free by mammogram and clinical exam) of 50 patients with breast cancer. Complete MSP assays on the fluid

obtained from both breasts of a total of 50 cancer patients. If recovery of cells is not satisfactory, optimize conditions, add patients to the study to get results from approximately 200-300 samples of ductal fluid (2-3 ducts per breast X 50) from 50 individuals. Compare MSP results with cytopathological data , and histopathology of the resected tumor, on each sample.

We have been able to, to date, recruit more (3) patients with breast cancer, since several in the first 25 did not yield fluid by ductal lavage, and in some patients, cytology was unable to detect any cells. We will add to this group until we reach 25 women.

Months 18-30: Approach high-risk, tumor free women who attend the BOSS (breast ovarian surveillance service) clinic in Johns Hopkins, and other high-risk individuals to undergo this procedure. Accrual will be slower in this category until the minimal discomfort involved and potential benefit becomes a publicized fact. Enter 50 individuals into the study. Perform MSP on cells obtained from each ductal lavage, on a total of approximately 200 samples.

We have not entered women into the trial who are high risk, but cancer free.

Months 30 to 36: Complete comparison of MSP results to cytopathologic and histopathology data, and data obtained by DNA analysis of tumor tissue obtained after surgery. Write and communicate papers.

KEY RESEARCH ACCOMPLISHMENTS:

1) The clinical trial has been initiated. 25 women have undergone ductal lavage of both breasts- the tumored breast and the contralateral high-risk breast. Wash fluids have been stored. Ductal cells are being examined by the cytopathologist.
2) A multiplex quantitative methylation specific PCR assay has been developed for all five-marker genes, that include RASSF1A and HIN-1. The sensitivity and specificity of the test have been completed. Pilot tests have been completed to test sensitivity and specificity in ductal cells from patients with breast cancer and those with benign cytology.
3) Collection of cells from both breast cancer bearing and contralateral breast has been completed. Cytology has been performed. Cytology slides will now be used to test methylated gene markers in the cells.

REPORTABLE OUTCOMES:

Publications

Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, Buluwela L, Weitzman SA, Marks J, S. Sukumar. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. Cancer Res., 61:2782, 2001.

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(in press, July 1, 2004).

Presentations:

June 2004: ICMIC Center in Johns Hopkins "Intraductal approach to breast cancer prevention, therapy and early detection"

June, 2004: National Cancer Institute

April, 2004: University of Alabama at Birmingham

April 2004: The Normal Breast Think Tank, Santa Barbara, CA

April 2004: AACR meetings, Orlando, Florida.

June 12, 2003, Howard/Hopkins Partnership Steering Committee Meeting, Howard University Cancer Center, Washington, D.C. "Comparative Gene Expression Analysis in African American and Caucasian Breast Cancer"

March 27-30, 2003, 3rd International Santa Barbara Symposium, The Intraductal Approach to Breast Cancer, Santa Barbara, California, "Detection of Breast Cancer Cells in Ductal Lavage and Blood Using Hypermethylated Gene Markers"

January 7-8, 2003, Institute of Medicine Committee on New Approaches to Early Detection and Diagnosis of Breast Cancer. Workshop on New Approaches to Breast Cancer Detection, Washington, D.C., "The Search for Breast Cancer Biomarkers"

January 22-23, 2003, National Council for Johns Hopkins Medicine, Meeting and Reception, Palm Beach, Florida, "Breast Cancer Research – Dawn of a New Era"

September 25–28, 2002 ,Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, Florida, "Early Detection of Breast Cancer by Molecular Anaylsis of Ductal Lavage Fluid"

September 19 – 21, 2002, Mildred Scheel Cancer Conference, Frankfurt, Germany, "Gene expression profiling of breast cancer – from bench research to early detection markers"

Patent application – Aberrantly Methylated Genes as Markers of Breast Malignancy (Docket # JHU1630; Ref. # DM-3729)

Collaborations: MD Anderson Cancer Center- Dr. Henry Keurer and Savitri Krishnamurthy- NAF analysis
MD Anderson Cancer Center MD- Dr. Banu Arun- Plasma analysis for methylation in high risk women
University of Kansas: Dr. Carol Fabian, Methylation markers for detection of tumor cells

in fine needle aspirates.

CONCLUSIONS:

Cells obtained by ductal lavage will prove to be valuable resource for detecting breast cancer cells early. Recognizing the paucity of the cells, a new method (QM-MSP) has been developed that will increase the specificity and sensitivity of detection of methyalted genes. This method may prove to be a PAP test for the breast.

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APPENDICES:

Appendix 1: Fackler, MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak, Sukumar S, Argani P. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int. Journal of Cancer 107, 970-975, 2003.

Appendix 2: Mehrotra J, Ganpat MM, Kaaan Y, Fackler MJ, McVeigh M, Lahti-Domenici J, Polyak, K, Argani P, Naab, T, Garrett E, Parmigiani G, Broome C and Sukumar S. ER/PR-negative breast cancers of young African American women have a higher frequency of methylation of multiple genes than those of Caucasian women. Clin Cancer Res. 10:2052-2057, 2004.

Appendix 3: Mehrotra J, Vali M, McVeigh M, Kominsky SL, Fackler MJ, Lahti-Domenici J, Polyak K, Sacchi N, Argani P, and Sukumar S. Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. Clin Cancer Res. 10: 3104-3109, 2004.

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Appendix 1

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DNA METHYLATION OF *RASSF1A, HIN-1, RAR-β, CYCLIN D2* AND *TWIST* IN *IN SITU* AND INVASIVE LOBULAR BREAST CARCINOMA

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Little is known about epigenetic silencing of genes by promoter hypermethylation in lobular breast cancers. The promoter methylation status of 5 cancer-related genes *(RASSF1A, HIN-1, RAR-β, Cyclin D2* and *Twist)* was evaluated in 2 types of lobular cancers, *in situ* (LCIS) and invasive lobular carcinomas (ILC) (n = 32), and compared to ductal *in situ* (DCIS) and invasive (IDC) breast cancers (n = 71). By using methylation-specific PCR (MSP), 100% of ILC and 69% of LCIS cases were found to have 1 or more hypermethylated genes among the panel of 5 genes (compared to 100% IDC and 95% of DCIS). Two or more hypermethylated genes were detected per tumor in 79% of invasive and 61% of *in situ* lobular carcinomas compared to 81% of IDC and 77% of DCIS. By contrast, DNA from nearly all normal reduction mammoplasty tissues (n = 8) was unmethylated for the 5 genes. The methylation profiles of lobular *vs.* ductal carcinomas with respect to *RASSF1A, Cyclin D2, RARβ,* and *Hin-1* genes were similar, suggesting that gene silencing by promoter hypermethylation is likely to be important in both groups of diseases. Distinctly different, *Twist* was hypermethylated less often in ILC (16%, 3/19 cases) than in IDC (56%, 15/27 cases) (p = 0.01). These results suggest that these 2 types of tumors share many common methylation patterns and some molecular differences. Additional studies might lend further understanding into the etiology and clinical behavior of this tumor type.

Key words: *methylation; breast cancer; lobular; ductal; DCIS; LCIS*

Comprehensive gene analyses such as serial analysis of gene expression (SAGE) and microarray analysis performed on breast cancer tissues revealed the expression profiles of thousands of genes and resulted in the identification of messenger RNAs that are over- and underexpressed in breast carcinomas compared to normal breast tissue.[1,2] We and others found that a major mode of tumor-specific downregulation of a number of these genes is by DNA hypermethylation.[3–8] The hypermethylation of cytosine residues in CpG-rich islands present in the promoter region of genes is an epigenetic alteration that leads to heritable changes in gene expression without changing the DNA sequence, most likely through the formation of repressive chromatin structure. Aberrant methylation of DNA is therefore believed to be an alternative pathway to cancer.[9] Studies of DNA hypermethylation in breast carcinoma have identified certain key genes as targets for epigenetic downregulation,[9–13] including receptors such as the *estrogen receptor*[6,14,15] and *retinoic acid receptor-β (RAR-β),*[5,16] cytokines, such as *HIN-1,*[17] cell signaling intermediates such as *RASSF1A,*[18,19] cell cycle regulators such as *Cyclin D2,*[4] adhesion molecules such as *E-cadherin,*[20,21] regulators of the actin cytoskeleton such as *gelsolin,*[8] DNA damage checkpoint genes such as *14.3.3 sigma,*[3,22] and sequence-specific transcription factors such as *HOXA5.*[23] Identification of these genes has helped begin to elucidate the molecular pathogenesis of breast carcinoma, as it reveals intracellular pathways that are altered that likely contribute to oncogenesis. In addition, identification of methylated genes provides a potential target for molecular detection of breast carcinoma, as even small amounts of methylated sequences are readily detectable by methylation-specific PCR (MSP).[24]

Because of the relatively low incidence of invasive lobular breast carcinoma (5–15% of invasive breast cancer,[25–28] though the incidence may be increasing[25–28]) and the fact that lobular carcinoma *in situ* does not typically form a tumor that can be identified grossly and sampled for molecular analysis,[28] characterization of the molecular alterations in this distinctive type of cancer is rudimentary at best. However, the few published studies suggest that there are differences in molecular alterations between invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC). For example, Mercapide *et al.* observed lower frequency of amplification of Cyclin D1 and HER-2/*neu* genes in ILC compared to IDC.[29] In addition, using comparative genomic hybridization (CGH), Gunther *et al.* found a significantly higher frequency of loss of chromosomes 16q, 17q and 22q and a lower incidence of chromosome 8q gain in ILC as compared to IDC.[30] Moreover, by immunohistochemistry, E-cadherin protein expression was found to be characteristically undetectable in both LCIS and ILC and intact in DCIS and IDC.[31,32] Most ILCs showed genetic or epigenetic changes affecting the E-cadherin *(CDH1)* gene.[33] In all cases in which there was loss of expression, there was biallelic inactivation of *CDH1* by promoter methylation, mutation or allelic loss in any combination. In a recent study, promoter hypermethylation of the Death-Associated Protein (DAP) Kinase gene was found to be significantly higher in ILC compared to IDC.[34] Therefore, it is likely that certain differing molecular alterations underlie the differing clinical and pathologic features of lobular and ductal breast cancers. For instance, LCIS is a marker of bilateral breast cancer risk, whereas DCIS is a direct, localized precursor to invasive carcinoma, and ILC is more likely to metastasize to the gastrointestinal tract than IDC.

With the exception of E-cadherin and DAP-kinase, the DNA methylation profile of the second most common type of breast cancer, lobular breast neoplasia, is currently unknown. Defining the methylation profiles of lobular cancers and comparing them to

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those obtained from ductal cancers could potentially help us distinguish between 2 major types of breast cancer at the molecular level. Moreover, determination of which genes are methylated in both types of cancer would aid in developing panels that will be most useful for detecting all breast cancers with a greater level of sensitivity.

In our present study, we examined the methylation profile of 32 lobular cancers, consisting of 13 LCIS and 19 ILC, by MSP analysis of the promoters of a panel of 5 genes: *RASSF1A, HIN-1, RAR-β, Cyclin D2* and *Twist*. Forty-four cases of DCIS and 27 of IDC were also studied and the results were compared. We now report that nearly all tumors contained one or more hypermethylated genes using this panel, with a few significant differences that are detailed herein.

MATERIAL AND METHODS

Case selection

Our study was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions. For normal breast tissue controls, 8 reduction mammoplasty specimens were used in which we confirmed benign findings by histologic examination. These patient ages ranged from 23–46 (mean 34 years). For both DCIS and LCIS, we selected a paraffin tissue block from cases of pure *in situ* carcinoma with no evidence of invasive carcinoma in the specimen or in prior biopsies of that breast. Cases of DCIS consisted of 14 grade 1, 12 grade 2 and 18 grade 3 lesions. Patient ages for the DCIS cases (n = 44) ranged from 34–79 (mean 54.5 years); patient ages for the LCIS cases (n = 13) ranged from 43–66 (mean 51.3 years). The IDC cases (n = 27) included 12 Elston grade 3 lesions, 14 grade 2 lesions, and 1 grade 1 lesion. Among the IDCs, data on *Cyclin D2, RAR-β* and *Twist* were available from a previous study[35] for 20 of the 27 tumors; MSP analysis of *RASSF1A* and *HIN-1* was performed in the course of the present study. Patient ages for the IDC cases ranged from 39–96 (mean 58.4 years). The ILC cases (n = 19) were all considered Elston grade 2 and patient ages ranged from 45–81 (mean 61.6 years).

Tissue sectioning and DNA extraction

The composition of the unstained slides from each archival formalin-fixed, paraffin-embedded tissue block studied was confirmed by histopathologic examination of surrounding hematoxylin and eosin (H & E)-stained sections. For each tumor, the lesion was identified on an initial H & E-stained section and confirmed to remain on a serial H & E section taken following preparation of unstained sections for nucleic acid extraction. Hence, the lesion was documented to be present on stained sections taken before and after preparation of the analyzed unstained sections. For DNA extraction, two 5 μm tissue sections were deparaffinized, scraped from the slide and extracted in 100 μl TNES (10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% SDS) containing 40 μg proteinase K for 16 hr at 50°C. The tissue extract was heat inactivated at 70°C for 10 min and clarified by centrifugation at 14K for 10 min; 50 μl of the supernatant was used directly as a source of DNA for sodium bisulfite treatment.

Sodium bisulfite treatment of DNA

Tissue and cell line DNAs were treated with sodium bisulfite and analyzed using methylation-specific PCR (MSP) as described by Herman *et al.*[24] This process converts nonmethylated cytosine residues to uracil, whereas methylated cytosines remain unchanged. All solutions were prepared fresh. Briefly, 50 μl DNA extract or 1 μg purified DNA (in 50 μl water) was incubated with 5.5 μl 2M NaOH for 10 min at 37°C. Subsequently 30 μl 10 mM hydroquinone and 520 μl 3M sodium bisulfite were added with mixing. The DNA was overlaid with 4 drops of oil and the sample was incubated 16 hr at 50°C. Bisulfite-modified DNA was purified using a Wizard DNA CleanUp System (Promega, Madison, WI) according to the manufacturer's instructions. DNA was eluted from the column in 50 μl sterile water into a 1.5 ml microfuge tube containing 5.5 μl 3 M NaOH, 2 μl glycogen and 16 μl 10 M ammonium acetate. DNA was precipitated with 200 μl absolute ethanol, washed twice with 70% ethanol, air dried and resuspended in 20 μl water at 0°C. Samples were aliquoted and stored at −80°C.

Methylation-specific polymerase chain reaction (MSP)

CpG islands in *Cyclin D2, RAR-β, Twist, HIN-1* and *RASSF1A* genes were examined by MSP. Forward and reverse primers were synthesized, which corresponded to the predicted sequence of methylated or unmethylated genomic DNA after sodium bisulfite treatment (Table III). For the reaction, 1 μl sodium bisulfite-treated DNA was added to 24 μl reaction buffer [1.25 mM dNTP, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO and 1.25 U RedTaq (Sigma, St. Louis, MO)] containing 100 ng each of forward and reverse primers specific to the unmethylated and methylated DNA sequences. Conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec, with a final extension cycle of 72°C for 5 min. The PCR products were resolved by electrophoresis in a 2% agarose gel, and the ethidium bromide-stained PCR products were imaged with the Eagle Eye II Video System (Stratagene, La Jolla, CA).

Statistical analysis

Statistical analyses were done with Stata 7.0 (Stata Corporation, College Station, TX). p-values were calculated using Fisher's exact test (2-sided). p-≤ 0.05 were considered statistically significant.

RESULTS

Frequency of hypermethylated genes in invasive lobular and ductal carcinoma

Methylation-specific PCR (MSP) was performed on DNA from 46 invasive mammary carcinomas, of which 19 were ILC and 27 were IDC. All invasive carcinomas (100%, 46/46) contained at least one hypermethylated gene in the panel (Table I, Fig. 1). In DNA from histologically benign, reduction mammoplasty specimens derived from normal women, only unmethylated alleles of the *HIN-1 RAR-β, Cyclin D2* and *Twist* and gene promoters were detected in 8 of 8 samples. A low-intensity methylated *RASSF1A* signal was detected in 1 of the 8 samples of breast tissue of normal women (Fig. 2). In the ILC cases, *RASSF1A* and *HIN-1* were hypermethylated at the highest frequencies. *RASSF1A* was hypermethylated in 16 of 19 (84%) cases and *HIN-1* in 15 of 19 (79%) cases. The high incidence of hypermethylated genes in ILC was similar to that found in IDC; 19 of 27 (70%) samples of IDC

TABLE I – FREQUENCY OF HYPERMETHYLATED GENES IN LOBULAR AND DUCTAL BREAST CANCER

	Incidence of hypermethylated/total no. tumor DNAs (%)				
Tissue	RASSF1A	Hin-1	RAR-β	Cyclin D2	Twist
LCIS	8/13 (62)	6/13 (46)	6/13 (46)	3/13 (23)	3/13 (23)
ILC	16/19 (84)	15/19 (79)	4/19 (21)	6/19 (32)	3/19 (16)[1]
DCIS-1	12/14 (88)	11/14 (79)	5/14 (36)	4/14 (29)	2/14 (14)
DCIS-2	7/12 (58)	7/12 (58)	7/12 (58)	3/12 (25)	3/12 (25)
DCIS-3	14/18 (78)	12/18 (67)	9/18 (50)	7/18 (39)	7/18 (39)
IDC	19/27 (70)	16/27 (60)	11/27 (41)	14/27 (52)	15/27 (56)[1]

[1]*Twist* IDC *vs.* ILC, p = 0.01 by Fisher's exact test (2-sided).

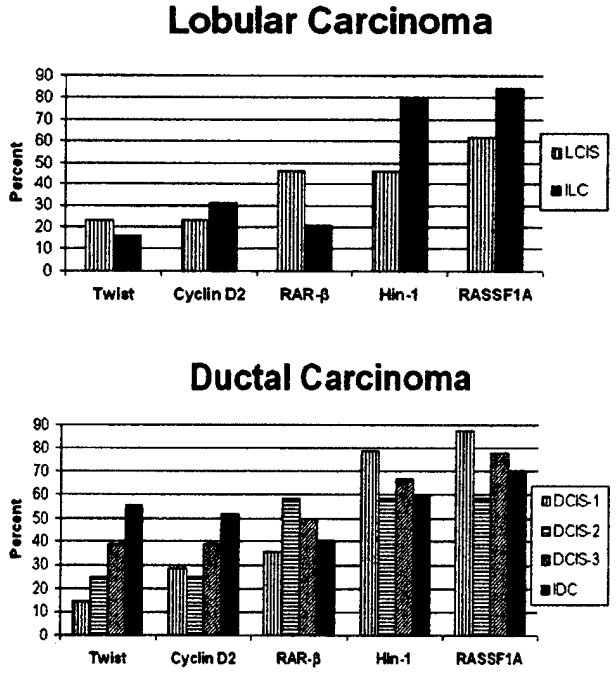

FIGURE 1 – Incidence of methylation in ductal and lobular breast cancer. The frequency of promoter hypermethylation within our gene panel was evaluated to compare *in situ vs.* invasive lobular (top panel) and ductal (lower panel) cancers. Top panel: percentage of cases methylated in LCIS and ILC. Lower panel: percentage of cases methylated in DCIS nuclear grades 1–3 (DCIS-1, DCIS-2, DCIS-3) and IDC.

contained hypermethylated *RASSF1A,* whereas 16 of 27 (60%) contained hypermethylated *HIN-1* (Table I, Figs. 1 and 2). However, a significant difference in the frequency of *Twist* hypermethylation was observed between ILC and IDC: the *Twist* gene promoter was hypermethylated at a much lower frequency of 16% (3 of 19) in ILC compared to 56% (15 of 27) in IDC ($p = 0.01$). *Cyclin D2* and *RAR-β* were hypermethylated at lower frequencies in ILC; 32% (6 of 19) of ILC contained hypermethylated *Cyclin D2,* whereas 21% (4 of 19) of ILC contained hypermethylated *RAR-β.* These latter 2 results were not significantly different from those seen in IDC, where 52% of cases (14 of 27) contained hypermethylated *Cyclin D2,* whereas 41% of cases (11 of 27) showed hypermethylated *RAR-β* (Table I). Hence, overall the methylation profile for the gene panel in ILC was highly similar to that seen in IDC. The prominent difference between the 2 tumor types was the lower incidence of hypermethylation in the *Twist* gene in ILC (Table I).

High incidence of hypermethylated genes in carcinoma in situ *(LCIS and DCIS)*

MSP analyses were performed on 13 LCIS and 44 DCIS cases of varied nuclear grade (Table I, Figs. 1 and 2). Analysis of the 5 genes showed one or more hypermethylated genes among *RASSF1A, HIN-1* and *RAR-β, Cyclin D2* and *Twist* in 69% (9 of 13) of LCIS and 95% (42 of 44) of DCIS.

In LCIS, *RASSF1A, HIN-1* and *RAR-β* genes were methylated with the highest frequency: 62% (8 of 13) for *RASSF1A,* 46% (6 of 13) for *HIN-1* and 46% (6 of 13) for *RAR-β* (Table I). These frequencies were similar to those seen in DCIS: 75% (33 of 44) for *RASSF1A,* 68% (30 of 44) for *HIN-1,* whereas 48% (21 of 44) for *RAR-β* (Table I, Figs. 1 and 2). *Cyclin D2* (23%, 3 of 13 cases) and *Twist* (23%, 3 of 13 cases) genes were methylated at the lowest frequency in LCIS. These results again paralleled those found in

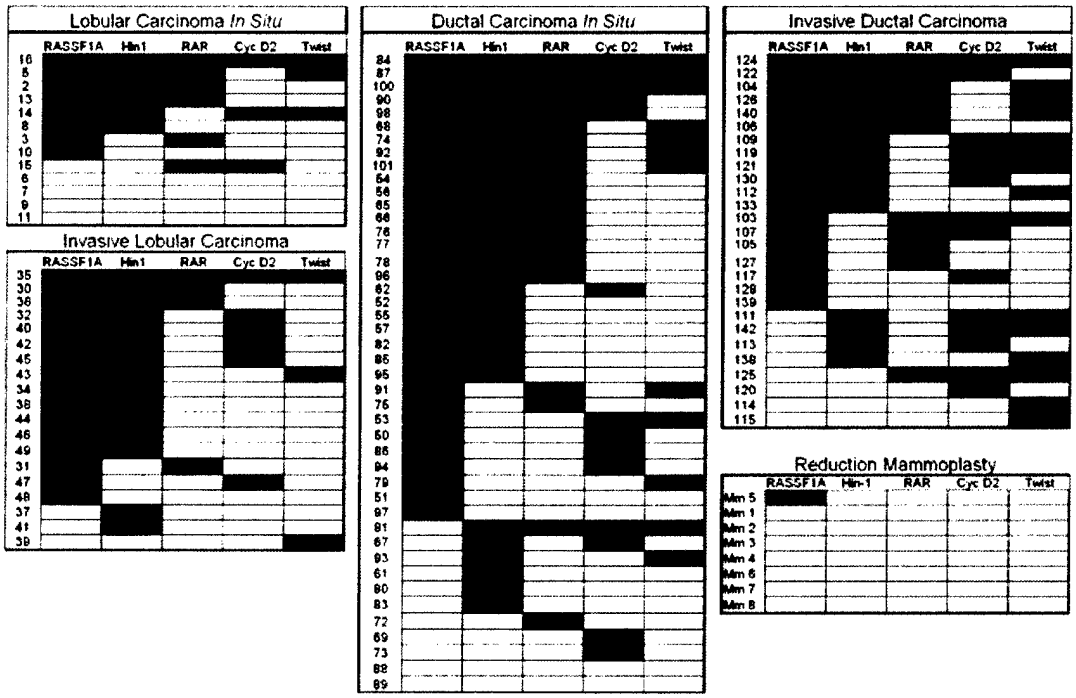

FIGURE 2 – Methylation profile of breast cancers for 5 genes. One hundred three cases of breast carcinoma were evaluated by MSP for the presence of promoter hypermethylation in the *RASSF1A, Hin-1, RAR-β, Cyclin D2 (Cyc D2)* and *Twist* genes. Carcinomas were grouped according to disease: LCIS (lobular carcinoma *in situ*; top left), ILC (invasive lobular carcinoma; lower left), DCIS (ductal carcinoma *in situ*; center panel top, middle and bottom, respectively, for nuclear grades 1–3) and IDC (invasive ductal carcinoma; top right). For comparison, 8 cases derived from normal patients undergoing reduction mammoplasty were similarly evaluated (bottom right). Results were scored as methylated (dark box) or unmethylated (light box).

DCIS, where overall, 32% of cases (14 of 44) contained hypermethylated alleles of *Cyclin D2* and 27% (12 of 44) of *Twist*.

In the frequency of hypermethylation of any gene in the panel, LCIS showed no statistically significant differences from DCIS. Similarly, LCIS and ILC displayed no significant differences in frequency of hypermethylated genes (Table I). A few differences became apparent when analyzing the ductal neoplasms. Although not statistically significant, it was of interest to note that the incidence of methylation in the *Twist* gene was higher in high nuclear grade DCIS: 14% (2 of 14) for grade 1, 25% (3 of 12) for grade 2 and 39% (7 of 18) for grade 3. In addition, the frequency of methylated *Twist* genes in low-grade DCIS of nuclear grades 1–2 (19%, 5 of 26) was significantly lower than in invasive carcinoma (56%, 15 of 27) ($p = 0.01$) (Table I).

Multigene promoter hypermethylation is frequent in both invasive breast cancers and in carcinoma in situ

Multigene promoter hypermethylation was a prominent feature of each group of breast cancer examined (Table II, Fig. 2). We found 42% (8 of 19) of ILC, 38% (5 of 13) of LCIS, 59% (16 of 27) of IDC and 48% (21 of 44) of DCIS had hypermethylated alleles of 3 or more of the 5 genes. These results suggest that global changes in gene promoter hypermethylation occur early in the evolution of cancer.

Specificity of promoter hypermethylation for carcinoma

To be a useful marker, gene promoter methylation should be specifically detected in carcinoma and not present in the adjacent normal ducts or stroma. To test this idea, we examined 9 pairs of invasive ductal carcinoma and adjacent normal tissues for evidence of promoter hypermethylation of Twist, Cyclin D2 and RAR-β (representative data in Fig. 3). We found at least one gene methylated in each of these tumors. All 9 histopathologically normal adjacent tissues contained only unmethylated Twist, Cyclin D2 and RAR-β.

DISCUSSION

Our studies establish that promoter hypermethylation of *RASSF1A, HIN-1, RAR-β, Cyclin D2* and *Twist* genes are frequent tumor-specific events in both *in situ* and invasive lobular carcinoma as well as in ductal breast carcinoma. All 5 genes are postulated to play a significant role in processes that can alter cellular proliferation in the breast, making them plausible tumor suppressor genes. The focus of our present study was to establish a molecular methylation profile for lobular cancers with respect to the 5 genes and to determine whether there were identifiable differences between the profiles of lobular *vs.* ductal carcinoma. We identified many similarities between these 2 tumor types and also found a distinct difference in the *Twist* gene, which was methylated less frequently in ILC *vs.* IDC.

Twist belongs to the basic-helix-loop-helix family of transcription factors and is implicated in lineage-specific cellular differentiation[36] and survival.[37] *Twist* has been postulated to be an oncogene that inhibits apoptosis in a p53-independent manner.[37] *Twist* may also alter cellular growth via its effects upon chromatin structure because it can directly inhibit histone acetyltransferase p300 and PCAF proteins.[38] We report here that *Twist* methylation is significantly lower in ILC than in IDC (Table I). *Twist* thereby joins *DAP Kinase, E-cadherin, Cyclin D1* and HER-2/*neu* as genes that are frequently altered differently in IDC as compared to ILC. It is likely that such genetic differences underlie the differing morphology and clinical features of these tumor types,[28,39] though the relative contributions of each genetic difference remain unknown.

Cyclin D2 is a member of the D-type cyclins, which are implicated in cell cycle regulation, differentiation and malignant transformation.[40] The *Cyclin D2* gene promoter is hypermethylated in 32% of ILC (Table I), a number that is consistent with previous and current findings in ILC and IDC.[4,34,41] LCIS has a similarly low frequency of *Cyclin D2* hypermethylation as DCIS. We noted a trend towards increased *Cyclin D2* methylation progressing from low-grade DCIS (grades 1 and 2, 27%) to high-grade DCIS (39%) to IDC (52%), though this did not reach significance in our study. This trend is consistent with the results previously reported by Lehmann *et al.*,[41] who found that increased *Cyclin D2* methylation correlated with increased Van Nuys' grade in DCIS.

HIN-1 is a recently identified putative cytokine discovered by SAGE to be highly expressed in the normal terminal ductal lobular unit (TDLU) and downregulated in invasive and *in situ* carcinomas by promoter hypermethylation.[17] *HIN-1* is considered a potential tumor suppressor based on studies demonstrating that its overexpression is growth inhibitory in breast cancer cell lines.[17] *HIN-1* mRNA expression was previously shown to be lost in IDC, and promoter hypermethylation was implicated as one mechanism of its loss.[17] The results of our current study are in concordance with these findings (Table I). We found a high incidence of *HIN-1* hypermethylation in ILC (79%) and in IDC (60%), as well as in DCIS (68% average of all grades). There were no significant differences between grades of DCIS. *HIN-1* was also very frequently hypermethylated in LCIS (46%). The high frequency of hypermethylated genes in *in situ* carcinoma provides strong evidence that *HIN-1* is epigenetically altered very early in the evolution of lobular as well as ductal carcinoma. It would be of interest to examine even earlier lesions of atypical hyperplasia and also normal-appearing lobules adjacent to the carcinoma to investigate if this alteration occurs at an even earlier stage.

RAR-β is also epigenetically silenced in many cancers, including breast, gastric, prostate and lung carcinomas. *RAR-β* is involved in regulation of cellular growth inhibition and apoptosis by mechanisms that are unknown. It is thought that *RAR-β* mediates the growth-inhibitory effects of retinoic acids upon breast cancer cells.[42–44] Our study established that the *RAR-β* promoter is also hypermethylated frequently in lobular carcinoma, both in LCIS (46%) and in ILC (21%) (Table I). Ductal carcinoma followed a similar profile, with 48% of DCIS and 32% of IDC demonstrating *RAR-β* methylation.

Epigenetic inactivation of *RASSF1A* is known to be widespread in carcinomas of lung, ovary, bladder, kidney and breast.[18,45–49] The function of *RASSF1A* is thought to involve regulation of Ras-like GTPases, due to interactions at the Ras-Rab association domain, a region homologous with the Ral GDS superfamily.[46,50] In our present study, the *RASSF1A* gene promoter was hypermethylated with the greatest frequency (averaging 74%, 76/103) of any of the 5 genes examined, an incidence that was similar between lobular and ductal, *in situ* and invasive breast cancers (Table I). These results are consistent with previous findings.[34,41] Although *RASSF1A* hypermethylation may be highly frequent in carcinomas, our study suggests that *RASSF1A* may also be methylated in certain benign conditions, possibly at low levels. We found trace levels of *RASSF1A* hypermethylation in 1 of our 8 normal reduction mammoplasty samples (Fig. 2). Interestingly, *RASSF1A* was not detectably hypermethylated in the contralateral benign breast tissue of the same patient. We also recently detected low-level *RASSF1A* promoter hypermethylation in a sample from an excisional biopsy that contained florid usual type ductal hyperplasia (data not shown). Consistent with our results, Lehmann *et al.*[41] recently reported epigenetic inactivation of *RASSF1A* expression in benign diseases like usual ductal hyperplasia and papillomas, though inactive normal breast epithelium and proliferating lactating epithelium was unmethylated. Dammann *et al.*[19] also found *RASSF1A*

TABLE II – FREQUENCY OF TUMORS CONTAINING MULTIGENE HYPERMETHYLATION OF *RASSF1A, HIN-1, CYCLIN D2, TWIST* AND/OR *RAR-β* IN LOBULAR AND DUCTAL, *IN SITU* AND INVASIVE CARCINOMAS

	% tumors with multigene methylation					
	No. genes hypermethylated					
	≥ 0	≥ 1	≥ 2	≥ 3	≥ 4	5
LCIS ($n = 13$)	31	69	61	38	15	8
ILC ($n = 19$)	0	100	79	42	0	5
DCIS ($n = 44$)	5	95	77	48	23	7
IDC ($n = 27$)	0	100	81	59	33	4

TABLE III – PRIMER SEQUENCES USED IN MSP ANALYSES

Gene	Direction	Sequence	bp
Unmethylated			
Cyclin D2	Forward	5′-AGA GTA TGT GTT AGG GTT GAT T	106
	Reverse	5′-ACA TCC TCA CCA ACC CTC CA	
RAR-β	Forward	5′-GGA TTG GGA TGT TGA GAA TGT	163
	Reverse	5′-CAA CCA ATC CAA CCA AAA CAA	
Twist	Forward	5′-TTT GGA TGG GGT TGT TAT TGT	193
	Reverse	5′-CCT AAC CCA AAC AAC CAA CC	
RASSF1A	Forward	5′-GGT TGT ATT TGG TTG GAG TG	180
	Reverse	5′-CTA CAA ACC TTT ACA CAC AAC A	
Hin-1	Forward	5′-GGT ATG GGT TTT TTA TGG TTT GTT	136
	Reverse	5′-CAA AAC TTC TTA TAC CCA ATC CTC A	
Methylated			
Cyclin D2	Forward	5′-GGC GGA TTT TAT CGT AGT CG	101
	Reverse	5′-CTC CAC GCT CGA TCC TTC G	
RAR-β	Forward	5′-GAA CGC GAG CGA TTC GAG T	142
	Reverse	5′-GAC CAA TCC AAC CGA AAC G	
Twist	Forward	5′-TTT CGG ATG GGG TTG TTA TC	200
	Reverse	5′-AAA CGA CCT AAC CCG AAC G	
RASSF1A	Forward	5′-GTT GGT ATT CGT TGG GCG C	160
	Reverse	5′-GCA CCA CGT ATA CGT AAC G	
Hin-1	Forward	5′-GGT ACG GGT TTT TTA CGG TTC GTC	136
	Reverse	5′-AAC TTC TTA TAC CCG ATC CTC G	

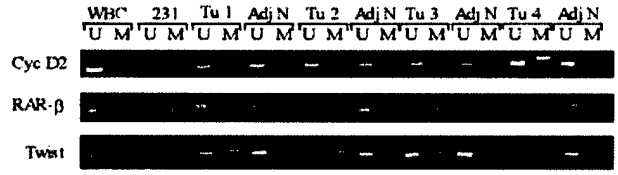

FIGURE 3 – Analysis of paired tumor and adjacent normal breast tissue. MSP analyses were performed on 4 pairs of tumor (Tu) and adjacent normal (Adj N) breast tissues using primers that specifically amplify *Cyclin D2 (Cyc D2), Retinoic Acid Receptor beta (RAR-β)* and *Twist* gene promoters. Peripheral blood (WBC) and MDA MB231 (231) cells were used as controls for detection of unmethylated (U) and hypermethylated (M) genes, respectively. The resulting PCR product was visualized with ethidium bromide after electrophoresis in 2% agarose.

hypermethylation in 7.5% of normal breast specimens. Hence, as Lehmann *et al.* have stated, low levels of *RASSF1A* hypermethylation may reflect nonphysiologic proliferation in the breast that does not qualify morphologically as carcinoma. Whether such proliferations are, in fact, of higher clinical risk than is suggested by their morphology remains to be determined. In the future, quantitative analysis of *RASSF1A* gene hypermethylation may be more useful for distinguishing benign from malignant proliferations.

Multigene methylation was a prominent feature of every group of breast cancer examined. Seventy-eight percent (79/103) of the tumors examined in our study had 2 or more of the 5 genes in our panel hypermethylated; 49% (50/103) of the tumors had 3 or more hypermethylated genes (Table II and Fig. 2). Since normal mammoplasty samples (Fig. 2) and normal tissues (ducts and stroma) adjacent to tumors (Fig. 3), are largely unmethylated, multigene methylation appears to be tumor specific.

We found a lack of any detectable methylation among the five genes examined in 31% (4/13) of LCIS and 5% (2/44) of DCIS cases (Table II and Fig. 2). In contrast, 0% (0/46) of invasive lobular and ductal carcinoma lacked detectable promoter hypermethylation (Table II and Fig. 2). The clinical manifestations of LCIS may hold an explanation for this difference. LCIS is generally considered to be a generalized risk factor for breast cancer, not a direct precursor,[28,39] and therefore, the lower frequency of genetic alterations in LCIS as a whole is logical. DCIS is thought to be a direct precursor to invasive carcinoma, though not all DCIS will progress to invasion. One could postulate that carcinoma *in situ* lesions containing unmethylated or less methylated genes may be less likely to invade and therefore less aggressive therapy for them would be indicated. Conversely, cases of carcinoma *in situ* with multiple hypermethylated genes may be at higher risk and require more aggressive therapy to prevent progression to invasive carcinoma. Future prospective studies will be needed to determine whether these gene markers are useful for risk assessment.

In summary, we have determined that *RASSF1A, Hin-1, RAR-β, Cyclin D2* and *Twist* genes are frequently hypermethylated in both preinvasive *(in situ)* and invasive lobular breast cancers in addition to ductal carcinomas. The *Twist* gene data suggest that underlying differences between lobular and ductal carcinomas exist, and the overall similarities in methylation profiles of *RASSF1A, RAR-β, Cyclin D2* and *Hin-1* between lobular and ductal breast cancer suggest that epigenetic silencing of important regulatory genes is a feature common to both groups of breast cancer. Based on these findings it appears that therapeutic intervention intended to reverse methylation-induced silencing of these genes may work equally well in early lobular and ductal carcinomas. These results also suggest that this panel of genes, capable of identifying nearly all breast carcinomas, could be useful as markers for early detection of breast carcinoma cells in ductal lavage and blood specimens.

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Estrogen Receptor/Progesterone Receptor-Negative Breast Cancers of Young African-American Women Have a Higher Frequency of Methylation of Multiple Genes than Those of Caucasian Women[1]

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ABSTRACT

Purpose: **To provide a molecular rationale for negative prognostic factors more prevalent in African-American (AA) than Caucasian (Cau) women, we investigated the frequency of promoter hypermethylation in invasive ductal breast cancers in the two races.**

Experimental Design: HIN-1, Twist, Cyclin D2, RAR-β, **and *RASSF1A* were analyzed in DNA from 67 AA and 44 Cau invasive ductal breast cancers, stratified by age and estrogen receptor/progesterone receptor (ER/PR) status, by methylation-specific PCR. Hierarchical multiple logistic regression analysis was applied to determine estimated probabilities of methylation. Expression of *HIN-1* mRNA was analyzed by *in situ* hybridization and quantitative reverse transcribed PCR.**

Results: **Significant differences between races were observed in the ER−/PR−, age < 50 subgroup; AA tumors had higher frequency of methylation ($P < 0.001$) in four of five genes as compared with Cau and also a higher prevalence (80 *versus* 0%; $P < 0.005$) of three or more methylated genes per tumor. No differences in gene methylation patterns were observed across the two races for ER+/PR+ tumors in all ages and ER−/PR− tumors in age > 50. ER+/PR+ status was associated with higher frequency of methylation in Cau tumors of all ages but only with the age > 50 subgroup in AA. Frequent *Cyclin D2* methylation was significantly associated ($P = 0.01$) with shorter survival time.**

Conclusion: **ER−/PR−, age < 50 tumors in AA women, have a significantly higher frequency of hypermethylation than in those of Cau women. Comparative studies, such as these, could provide a molecular basis for differences in tumor progression and pathology seen in the two races.**

INTRODUCTION

Among different racial groups within the United States, overall breast cancer incidence is highest in Caucasian (Cau) women followed by African-American (AA) women (1). Compared with Cau, AA women in the <50 years age group have a higher incidence of breast cancer, whereas in the >50 age group, incidence is lower (1). However, mortality attributable to breast cancer in all age groups is higher in AA than Cau and higher than in all races (1). Negative prognostic factors for survival, which are more prevalent in AA than Cau women, are late stage at diagnosis (increased tumor size, node positive, and more distant metastasis), early onset disease (<50 years age), estrogen receptor negative (ER−) progesterone receptor negative (PR−), poorly differentiated tumors, high S phase and mitotic index, obesity, and more adverse p53 mutations (2). There is sparse literature on the molecular basis for these clinico-pathological differences in breast cancer between the two races. Unique inherited *BRCA1* and *BRCA2* (3) and *p53* mutations (2), *H-ras-1* and (4) *CYP1A1* polymorphisms (2), and overexpression of *Cyclin D1* (5) have been associated more strongly with AA than Cau breast cancer.

Loss of gene expression by promoter hypermethylation has been shown to have clinical implications in some cancers (6, 7). Hypermethylation-mediated loss of gene expression could provide the cell with growth-promoting characteristics, such as insensitivity to antigrowth signals, self-sufficiency in growth signals, limitless replicative potential, and evasion of apoptosis (8). *HIN-1,* a putative cytokine, has a role in regulating the proliferation and differentiation of normal luminal mammary epithelial cells (9). *HIN-1* methylation is reported in 74% of primary breast carcinomas and ~100% of ductal carcinoma *in situ* (9, 10), where its expression is undetectable. Almost 40% of primary breast cancers and ~28% of *in situ* cancers are methylated for *Twist,* a gene implicated in apoptosis (8, 10, 11). *Cyclin D2* is involved in cell cycle regulation. It is methylated in 50% of primary breast carcinomas and ductal carcinoma *in situ,* suggesting that loss of its expression is an early event in tumorigenesis (10, 12). *RASSF1A,* a putative tumor suppressor

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gene, is methylated in 50–60% of primary breast carcinomas (10, 13) and associated with poor survival in non-small cell lung cancer patients (13, 14). Retinoic acid receptor (*RAR*)-β functions in inhibition of proliferation, apoptosis, and senescence (8) and is methylated in ~50% of invasive and *in situ* breast cancers (10, 15).

Identifying race-specific molecular markers could lead to better understanding of the differences in the etiological factors contributing to the development of breast cancer between the two populations and also optimal clinical management and therapeutic intervention. As a first step toward this goal, epigenetic alterations of DNA promoter hypermethylation were analyzed in AA and Cau breast cancers in a panel of five genes frequently hypermethylated in breast cancer: (*a*) *HIN-1* (9); (*b*) *Twist* (11); (*c*) *Cyclin D2* (12); (*d*) *RAR*-β (*RAR*-β P2 promoter; Refs. 15 and 16); and (*e*) *RASSF1A* (13). Hypermethylation and loss of expression of these genes have been detected in invasive breast cancer but not in normal mammary epithelial cells, mammary stroma, and WBCs. Differences in DNA methylation in tumors in subgroups stratified by race, ER/PR status, and age are discussed.

Table 1 Patient and tumor characteristics

	AA[a]	Cau	P^b
	n (%)	*n* (%)	
Age (yrs)			0.70
>50	38 (57)	23 (52)	
<50	29 (43)	21 (48)	
ER/PR status			0.56
Positive	30 (45)	23 (52)	
Negative	37 (55)	21 (48)	
Elston grade			0.54
I	2 (3)	3 (7)	
II	30 (45)	17 (39)	
III	35 (52)	24 (55)	
Stage			0.40
1	8 (12)	3 (7)	
2	24 (36)	22 (50)	
3	31 (46)	19 (43)	
Unknown	4 (6)	0 (0)	
Lymph node status			0.046
Positive	33 (49)	32 (73)	
Negative	29 (43)	12 (27)	
Unknown	5 (7)	0 (0)	

[a] AA, African-American; Cau, Caucasian; ER, estrogen receptor; PR, progesterone receptor.
[b] *P* determined by Fisher's exact test.

MATERIALS AND METHODS

Tissues. Paraffin-embedded blocks and frozen tissues (67 AA and 44 Cau) were obtained from the Surgical Pathology Department of the Johns Hopkins Hospital and Howard University Cancer Center after approval by the human investigations committees from both institutions. All breast tumors were invasive ductal breast cancers except for two AA samples, which were mixtures of invasive ductal breast cancer and invasive lobular carcinoma.

DNA Extraction and Methylation-Specific PCR (MSP). DNA was extracted (10) from fixed or frozen sections (after a 30-s wash in 70% ethanol, followed by water and air drying), and MSP analysis on sodium bisulfite-treated DNA was performed as described (10, 17). The primer sequences used are described earlier (10). MSP analysis for ≥10 samples was performed in both the Hopkins and Howard University laboratories to control for any technical bias.

mRNA analysis by Quantitative Reverse Transcribed-PCR and *in Situ* Hybridization. The following primer and probe sets were used to amplify cDNA: *HIN-1,* forward, 5′CATGAAGCTCGCCGCCCT3′; reverse, 5′CTTGGCCGAGCCCACTAAG3′; probe, FAM-CTCTGCGTGGCCCTGTCCTGCA-TAMRA; *glyceraldehyde-3-phosphate dehydrogenase,* forward, 5′CCCATGTTCGTCATGGGTGT3′; reverse, 5′-TGGTCATGAGTCCTTCCACGATA-3′; probe, TET-CTGCACCACCAACTGCTTAG-TAMRA. The comparative threshold cycle method was used to analyze expression in different tissue samples (18). The experimental data were normalized to the internal *glyceraldehyde-3-phosphate dehydrogenase* control and normal breast tissue (at surgical margins of the tumors). *In situ* hybridization was performed for *HIN-1* as described previously (19).

Statistical Analysis. Fisher's exact test was used to compare categorical variables across race. Standard logistic regression analyses were performed to test the association of important risk factors, *i.e.,* lymph node, stage, and grade with methylation. The Cox proportional hazards model and Log-rank test were used for assessing associations between methylation and survival outcomes and patient characteristics and survival outcomes. In this study, we consider many hypothesis tests. Therefore, we adopt a more stringent α cutoff of 0.005 for reporting significant results (*i.e.,* significance is determined by $P \leq 0.005$). To examine whether methylation patterns differ by race within subgroups defined by age and ER/PR status, we used a multilevel model, similar to a multiple logistic regression but fits the logistic model to all genes simultaneously, with the assumption that effects across genes are from a common distribution. Motivation (20–22) and implementation details (23) are given in the supplementary materials.[7] Results are summarized by estimates of methylation frequencies by group, 95% probability intervals on those, and posterior tail probabilities for comparisons across groups (these closely correspond with *P*s in this specific case).

RESULTS

MSP analysis was performed on DNA from 67 AA and 44 Cau primary invasive ductal breast cancer (Table 1; Fig. 1). Comparative prevalence of promoter hypermethylation of *HIN-1, Twist, Cyclin D2, RAR*-β, and *RASSF1A* genes and methylation of multiple (more than or equal to three) genes in AA and Cau breast carcinomas as stratified by ER/PR status and age are shown in Table 2.

We used multiple logistic regression analysis to assess the association of risk factors, such as lymph node status, tumor grade, and stage with methylation of the five genes. None of the risk factors showed significant association with methylation of

[7] http: cancerres.aacrjournals.org.

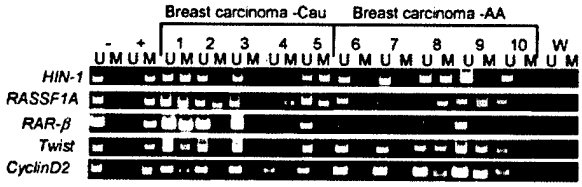

Fig. 1 Methylation-specific PCR analysis on each sample using unmethylated (*U*) and methylated (*M*) sequence-specific primers. DNA from WBCs (−) and MDA-MB-231 (+) breast cancer cell line served as negative and positive controls for methylated genes, respectively. Water (*W*) as negative control for both unmethylated and methylated genes. WBC also served as a positive control for unmethylated genes.

any gene (data not shown) except for *Twist* methylation, which showed a borderline association with stage 2/3 of breast cancer ($P = 0.03$). To assess the association between age (age < 50 *versus* >50), race (AA *versus* Cau), and ER/PR status (positive *versus* negative) and their interactions, a multilevel, multivariate logistic regression model showed that the main effect of age < 50, main effect of race, two-way interaction between race and ER/PR, and two-way interaction between age < 50 and ER/PR were constant across genes. The model showed that the following effects varied across genes: (*a*) the main effects of ER/PR; (*b*) two-way interaction between race and age < 50; and (*c*) three-way interaction between race, age < 50, and ER/PR. The results from the model are shown as estimated percentage of methylation for each gene and methylation of multiple (more than or equal to three methylated) genes in Fig. 2.

Major Differences between AA and Cau Breast Cancers Are in the Age < 50, ER−/PR− Subgroup. ER+/PR+ tumors in all age subgroups (age > 50 and <50) showed essentially no difference in estimated percentage of methylation across race for any of the five genes: (*a*) *HIN-1*; (*b*) *Twist*; (*c*) *Cyclin D2*; (*d*) *RAR*-β; and (*e*) *RASSF1A* (Fig. 2).

We found pronounced differences in an estimated percentage of frequency of methylation across race in the ER−/PR−, age < 50 subgroup (Fig. 2). Breast tumors in ER−/PR−, age < 50 AA women, had a significantly higher estimated percentage of methylation for four of five genes, as compared with those from Cau women in this subgroup. The estimated percentages of frequency of methylation were: (*a*) *HIN-1*, 79% in AA *versus* 19% in Cau ($P < 0.0001$); (*b*) *Twist*, 67% in AA *versus* 16% in Cau ($P < 0.0001$); (*c*) *Cyclin D2*, 64% in AA *versus* 19% in Cau ($P < 0.0001$); and (*d*) *RASSF1A*, 76% in AA *versus* 29% in Cau ($P < 0.0001$). We observed a similar trend for *RAR*-β with an estimated frequency percentage of methylation of 40% in AA *versus* 8% in Cau, although this difference was not statistically significant ($P = 0.01$). On the contrary, no race-related effects on methylation were observed in the ER−/PR−, age > 50 subgroup of tumors (Fig. 2).

Methylation As a Function of ER/PR Status and Age within Each Race. Looking at an estimated percentage of methylation patterns within the same race (Fig. 2), we observed that in the age > 50 subgroup, both Cau and AA tumors had significantly higher methylation for *HIN-1* and *RASSF1A* in the ER+/PR+ *versus* ER−/PR− tumors [Cau: *HIN-1*, 85 *versus* 38% ($P < 0.0001$); *RASSF1A*, 92 *versus* 51% ($P < 0.0001$); AA: *HIN-1*, 87 *versus* 50% ($P < 0.0001$); *RASSF1A*, 93 *versus* 63% ($P < 0.0001$)]. In the age < 50 subgroup, Cau tumors followed the same trend as seen above, and this effect was consistent in four of five genes [ER+/PR+ *versus* ER−/PR−: *HIN-1*, 84 *versus* 19% ($P < 0.0001$); *Cyclin D2*, 52 *versus* 19% ($P < 0.005$); *RAR*-β, 37 *versus* 8% ($P < 0.005$); *RASSF1A*, 91 *versus* 29% ($P < 0.0001$)]. The *Twist* gene showed a similar trend (44% in ER+/PR+ *versus* 16% in ER−/PR−), but this difference was not significant ($P = 0.02$). Surprisingly, AA tumors in the age < 50 subgroup did not show the same trend of higher methylation in ER+/PR+ tumors that was seen in the other subgroups. As evident in Fig. 2, ER−/PR− age < 50 tumors from AA women clustered with ER+/PR+ subgroups in all comparisons.

Methylation at Multiple Loci per Tumor. Using the gene-specific estimate described thus far, we also estimated prevalence of multiple (more than or equal to three) gene methylation in each subgroup (Fig. 2). All ER+/PR+ tumors

Table 2 Frequency of DNA methylation in AA[a] and Cau breast carcinomas as stratified by ER/PR status and age

	HIN-1	Twist	Cyclin D2	RAR-β	RASSF1A	≥3 genes/tumor
Subgroups	No. of tumors methylated/total no. tested (%)					
All						
Cau	24/44 (55%)	15/44 (34%)	18/44 (41%)	11/42 (26%)	32/44 (73%)	19/44 (43%)
AA	50/66 (81%)	33/64 (52%)	36/67 (54%)	22/65 (34%)	52/67 (78%)	42/67 (63%)
ER−/PR− > 50						
Cau	3/11 (27%)	3/11 (27%)	3/11 (27%)	4/9 (44%)	7/11 (64%)	3/11 (27%)
AA	9/22 (41%)	8/20 (40%)	13/22 (59%)	5/20 (25%)	15/22 (68%)	10/22 (45%)
ER−/PR− <50						
Cau	2/10 (20%)	2/10 (20%)	3/10 (30%)	0/10 (0%)	2/10 (20%)	0/10 (0%)
AA	14/15 (93%)	12/15 (80%)	9/15 (60%)	3/15 (20%)	11/15 (73%)	12/15 (80%)
ER+/PR+ > 50						
Cau	10/12 (83%)	7/12 (58%)	5/12 (42%)	3/12 (25%)	12/12 (100%)	8/12 (67%)
AA	15/16 (94%)	8/15 (53%)	9/16 (56%)	6/16 (38%)	14/16 (88%)	12/16 (75%)
ER+/PR+ <50						
Cau	9/11 (82%)	3/11 (27%)	7/11 (64%)	4/11 (36%)	11/11 (100%)	8/11 (73%)
AA	12/13 (92%)	5/14 (36%)	5/14 (36%)	8/14 (57%)	12/14 (86%)	8/14 (57%)

[a] AA, African-American; Cau, Caucasian; ER, estrogen receptor; PR, progesterone receptor.

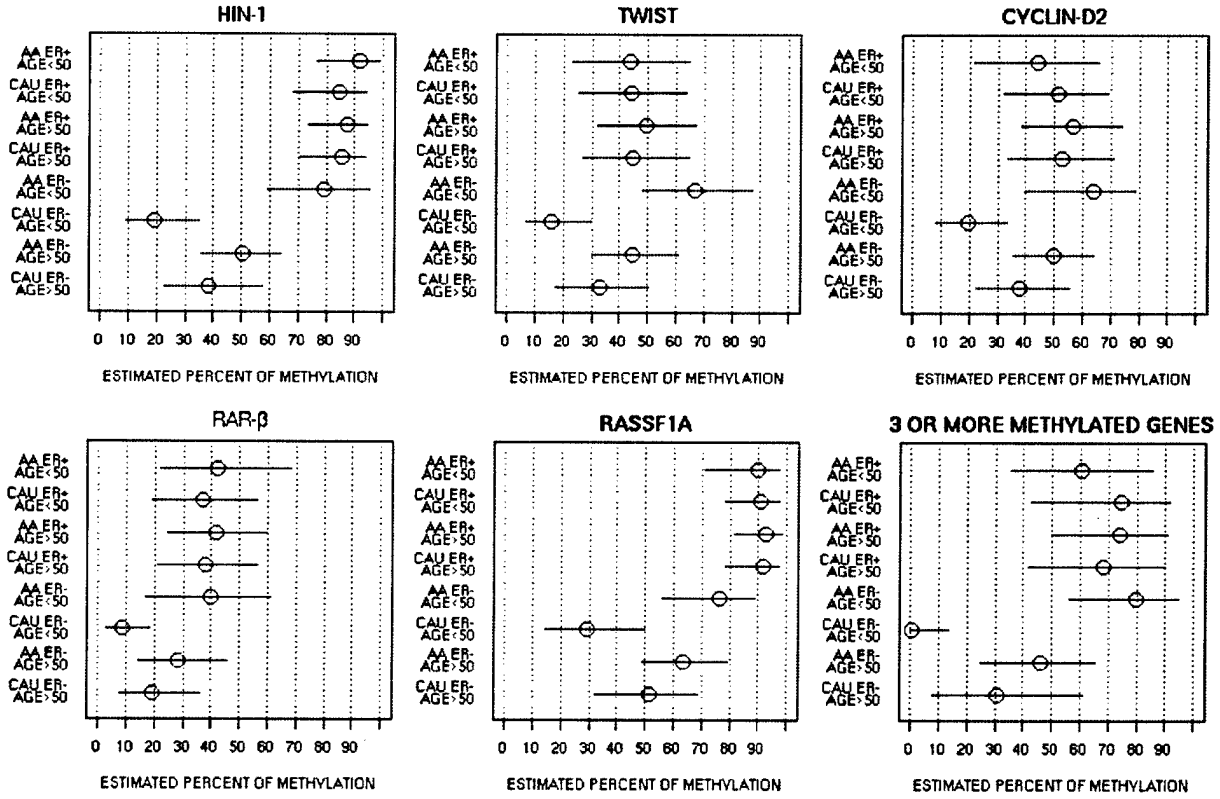

Fig. 2 Estimated percentage of methylation for promoter hypermethylation of *HIN-1, Twist, Cyclin D2, RAR-β*, and *RASSF1A* and methylation of more than or equal to three genes in African-American (*AA*) and Caucasian (*Cau*) breast cancers as stratified by race, estrogen receptor (*ER*)/progesterone receptor (*PR*) status, and age. The estimated percentage values and corresponding 95% probability intervals were derived from the multiple logistic regression analysis used.

had a higher prevalence of methylation at multiple loci. Across races, the most striking difference was again seen in the ER−/PR− age < 50 subgroup of tumors. AA tumors in this group had a very high proportion of tumors (estimated 80%) with multiple methylated genes as compared with Cau tumors (estimated 0%; $P < 0.005$). We computed odds ratios for the association between methylation of two genes and found highly significant associations between *HIN-1* and *RASSF1A, HIN-1* and *Twist,* and *Cyclin D2* and *Twist* methylation (data not shown). In addition, we noted that all pairwise odds ratios showed a positive association between methylation events, although not significant.

Hypermethylation of *HIN-1* Correlates with mRNA Expression in the Tumors. To assess the biological significance of hypermethylation in this study, as a representative, we analyzed the expression of *HIN-1* in primary tumors using quantitative reverse transcribed PCR. We analyzed 12 tumors that contained an unmethylated and 14 tumors that contained a methylated *HIN-1* gene. As predicted, compared with normal breast tissue, 10 of 14 tumors (71%) methylated for *HIN-1* gene showed no detectable expression of *HIN-1* mRNA (Fig. 3*A*). Of the remaining 4, 3 showed very low levels, and only 1 tumor, contrary to expectation, showed a high level of expression. Seven of 12 tumors (58%), unmethylated for *HIN-1,* showed detectable mRNA expression, whereas 5 did not. Not observing expression in all of the tumors in this group is consistent with previous findings that *HIN-1* expression is silenced in breast cancer, primarily by methylation of its promoter sequences, but other modes of transcriptional silencing are operative in tumors containing unmethylated *HIN-1* genes (9).

We further confirmed the results of the quantitative reverse transcribed PCR analysis of *HIN-1* expression by performing *in situ* hybridization on paraffin-embedded tissue sections. Six tumors unmethylated and five methylated for the *HIN-1* gene were analyzed by *in situ* hybridization (representative photomicrographs shown in Fig. 3*B*). Five of six (83%) unmethylated tumors were positive for *HIN-1* expression, whereas only two (40%) of five methylated tumors showed partial expression of *HIN-1* (<30% cells). Thus, using two independent approaches, we observed a direct correlation between *HIN-1* methylation and loss of mRNA expression.

Correlating Methylation with Clinical Outcome of Patients. We analyzed patient data to investigate whether frequency of methylation was associated with time of diagnosis to time to all cause death or time of diagnosis to time to cancer death (data not shown). We view these analyses as exploratory because: (*a*) our sample size was small; and (*b*) the presence of a potential confounding factor that the patients received a variety of treatments. In this data set, we found that tumor grade III and ER/PR status were associated with survival. We performed

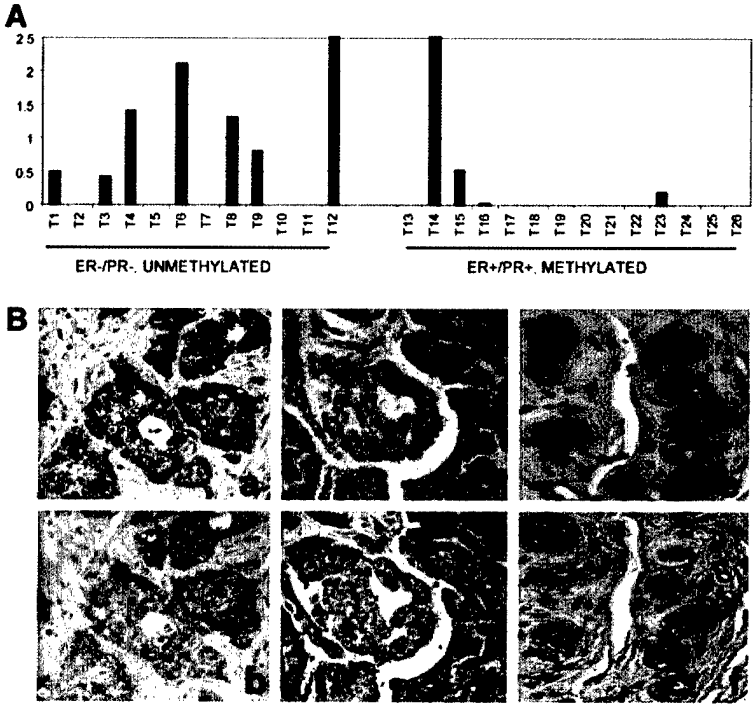

Fig. 3 *HIN-1* mRNA expression in primary breast cancers. *A*, quantitative reverse transcriptase PCR analysis of primary breast tumors. mRNA levels in the tumors, relative to the mean of mRNA levels in three normal breast tissues, are shown. *B*, *in situ* hybridization with *HIN-1* antisense (*a*, *c*, and *e*) and sense ribo-probes (*b*, *d*, and *f*) in tumors unmethylated (*a* and *b*) and methylated (*c* and *d*) for the *HIN-1* gene. Normal ducts (*e* and *f*) from the same section as *c* and *d* showing specific *HIN-1* expression with the antisense *HIN-1* probe (*e*). Magnification: ×200.

Cox proportional hazards model analyses, where tumor grade and ER/PR status were included as covariates. There was evidence of a significant association of cancer-related death (Hazard's ratio = 3.82, $P = 0.01$) with highly frequent methylation of *Cyclin D2* alone. Interestingly, in accord with these findings, multiple logistic regression analyses also showed that the estimated percentage of methylation of *Cyclin D2* gene was significantly ($P < 0.0001$) more prevalent in AA (64%) than Cau (19%) breast cancers in the age < 50, ER−/PR− subgroup of breast tumors (Fig. 2).

DISCUSSION

Survival of breast cancer patients is significantly worse in AA than Cau women for reasons not well understood at the molecular level. Hormone-negative receptors and early age of onset are negative prognostic factors that are more prevalent in AA than in Cau breast cancers (2). In this study, we investigated the hypothesis that breast cancers in AA and Cau women are characterized by many common but some distinct molecular alterations that result in altered patterns of gene expression and differences in clinical presentation and behavior. The more aggressive tumors and early onset breast cancer in AA women may be caused by specific alterations in gene expression patterns that are different or more pronounced in AA than Cau and could be initiated at different stages of tumor progression in the two races. As a first step toward testing this hypothesis, we compared molecular alterations at the epigenetic level by studying DNA promoter hypermethylation profiles of AA and Cau breast cancers for a panel of five genes known to be frequently hypermethylated in breast cancer: (*a*) *HIN-1*; (*b*) *Twist*; (*c*) *Cyclin D2*; (*d*) *RAR*-β; and (*e*) *RASSF1A*. Only ER−/PR− age < 50 tumors differed significantly in their methylation patterns in AA and Cau, providing evidence to support the hypothesis that early onset breast cancer and ER-negative breast carcinomas in Cau and AA women are different at the molecular level. A better understanding of such molecular alterations may potentially provide early diagnostic markers and drug targets for different groups of patients.

In this study, by examining the methylation patterns in tumors stratified by race, ER/PR status, and age, we could delineate the similarities and distinctions between different subgroups. Estrogen is a key factor in the development of normal mammary glands in women and significant player in the development and progression of breast cancer (24, 25). There is a preponderance of ER−/PR− tumors in AA women (2). ER status has been associated with methylation of *BRCA1*, the *ER*-α gene, and *HIN-1*. Hypermethylation of a tumor suppressor gene *BRCA1* has been reported in 11–32% of primary sporadic breast carcinomas (26–29), and methylation strongly correlated with lack of ER/PR receptor expression (26, 29). *BRCA1* methylation has also been found to be more strongly associated with the uncommon (<5%) medullary and mucinous subtypes of breast cancer (27), which some studies report are more frequent in AA cancers (30). A recent study observed that *HIN-1* methylation is significantly higher in ER+ breast tumors than ER− tumors (28). There is evidence in the literature that several genes undergo hypermethylation in subpopulations of normal cells as a function of older age, which increases during progression of colon cancer (31, 32). In breast cancer, we did not see an association of methylation with age or any of the other risk factors, such as lymph node status, tumor grade, and stage (data not shown). This suggests that, as demonstrated in colon cancer

by Yamashita *et al.* (33), one could question the existence of a methylator phenotype in breast cancer.

Our study was limited by the sample size to make any correlation of methylation with survival in the different subgroups. However, we did observe an association of *Cyclin D2* methylation with survival. *Cyclin D2* was significantly more methylated in ER−/PR− AA tumors as compared with their Cau counterparts.

In conclusion, by analyzing the hypermethylation profiles of five genes, we could decipher many significant disparities and some similarities between AA and Cau breast cancers. In the ER−/PR− <50 subgroup, higher methylation in AA breast cancers compared with Cau breast cancers is associated with negative prognostic factors (ER status and age) that are more prevalent in AA. This observed heterogeneity between AA and Cau breast cancers underscores the need for a more comprehensive comparison of breast cancers between these races, which could lead to better clinical management for different racial groups.

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Very High Frequency of Hypermethylated Genes in Breast Cancer Metastasis to the Bone, Brain, and Lung

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ABSTRACT

Purpose: **Most often it is not the primary tumor, but metastasis to distant organs that results in the death of breast cancer patients. To characterize molecular alterations in breast cancer metastasis, we investigated the frequency of hypermethylation of five genes (*Cyclin D2*, *RAR-β*, *Twist*, *RASSF1A*, and *HIN-1*) in metastasis to four common sites: lymph node, bone, brain, and lung.**

Experimental Design: **Methylation-specific PCR for the five genes was performed on DNA extracted from archival paraffin-embedded specimens of paired primary breast cancer and its lymph nodes (LN) metastasis ($n = 25$ each); in independent samples of metastasis to the bone ($n = 12$), brain ($n = 8$), and lung ($n = 10$); and in normal bone, brain, and lung ($n = 22$).**

Results: **No hypermethylation was detected in the five genes in the normal host tissues. In paired samples, LN metastasis had a trend of higher prevalence of methylation compared with the primary breast carcinoma for all five genes with significance for *HIN-1* ($P = 0.04$). Compared with the primary breast carcinomas, all five genes had higher methylation frequencies in the bone, brain, and lung metastasis, with *HIN-1* and *RAR-β* methylation being significantly higher ($P < 0.01$) in each group. Loss of expression of all five genes correlated, with a few exceptions, to hypermethylation of their promoter sequences in metastatic carcinoma cells microdissected from LNs.**

Conclusion: **The frequent presence of hypermethylated genes in locoregional and distant metastasis could render them particularly susceptible to therapy targeted toward gene reactivation combining demethylating agents, histone deacetylase inhibitors, and/or differentiating agents.**

INTRODUCTION

Metastatic breast cancer remains essentially incurable, and almost all women diagnosed will eventually die from their disease (1, 2). Therefore, important goals of current therapy have been to palliate symptoms and prolong patient survival. Molecular analysis of metastatic lesions is slowly leading to an understanding of the events underlying distant spread of breast cancer cells from the site of origin (1, 3, 4). Comparative large-scale gene expression analysis by SAGE (serial analysis of gene expression) of breast cancer and normal breast epithelial cells has led to the discovery of several genes that are differentially expressed between the two tissues (5–7). This knowledge might, in the near future, provide potent targets for therapy.

Work from our laboratory (8–10) and others (11–13) have shown that the genes that are expressed in normal breast epithelium but not in the carcinoma cells are frequently silenced by promoter methylation. Promoter hypermethylation is now recognized as a common method for cancer-specific repression of gene transcription. Some of the genes most frequently methylated (30–90%) in breast carcinomas, but not in normal breast epithelium are 14.3.3 sigma (8), *Cyclin D2* (9, 10, 14), *RAR-β* (the P2 promoter; Refs. 10, 11, 14), *Twist* (10, 14), *RASSF1A* (10, 15, 16), and *HIN-1* (10, 17). Our recent study has shown that hypermethylation of these genes is a feature common to both lobular and invasive breast carcinomas (10).

In this report, we investigated the incidence of methylation of the five genes, *Cyclin D2*, *RAR-β* (the P2 promoter), *Twist*, *RASSF1A*, and *HIN-1* in breast cancer metastasis in the lymph node (LN), bone, lung, and brain. We report that when compared with primary invasive ductal carcinomas, there is a striking increase in the incidence of tumor-specific methylation in breast cancer metastasis to all four sites. We demonstrate that hypermethylation of gene sequences correlates with loss of gene expression. The significance of these findings is discussed in the context of their therapeutic potential.

MATERIALS AND METHODS

Tissues. Paraffin-embedded samples of paired primary invasive ductal carcinomas of the breast and their LN metastasis; breast cancer metastasis to the bone, brain, and lung; and normal samples of bone, brain, and lung, and snap-frozen LNs containing metastatic cells were obtained from the Surgical Pathology archives of the Johns Hopkins Hospital after obtaining approval from the institutional review board. A hema-

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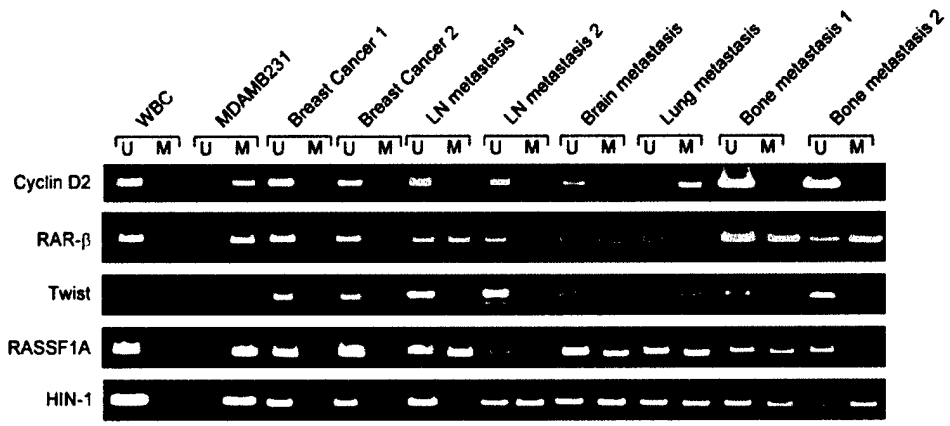

Fig. 1 Incidence of hypermethylation in primary breast cancer, local metastasis [lymph node metastasis (*LN metastasis*)], and distant metastasis (*Brain metastasis*, *Lung metastasis*, *Bone metastasis*). Methylation-specific PCR (MSP) analysis was performed using primers specifically amplifying *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* gene promoters. Peripheral WBCs (*WBC*) and MDA-MB231 cells served as controls for unmethylated (*U*) and hypermethylated (*M*) genes, respectively. PCR products were visualized using ethidium bromide after electrophoresis on a 2% agarose gel.

toxylin-eosin-stained section was examined for each tissue before entry into the study. The percentage of carcinoma cells in the tumor sections varied from 20 to 50%. Normal bone, brain, and lung tissues were obtained from surgeries for disease not related to cancer.

DNA Extraction. One to two 5-μm tissue sections were deparaffinized with xylene and scraped into tubes containing 100 μl of Tris/NaCl/EDTA/SDS plus proteinase K (10, 18). The tubes were incubated in a rotary shaker overnight at 50°C. The tubes were then heated at 65°C for 10 min to inactivate the enzyme, and were centrifuged at 14,000 rpm for 10 min. The supernatant was used directly for sodium bisulfite treatment (10, 18).

Bisulfite Treatment of DNA. Fifty μl of the cell lysate (see above) or 1 μg of purified DNA was treated with sodium bisulfite for 16 h as described previously (10, 18). After the DNA purification step, the DNA was resuspended in 20 μl of water. PCR was performed for each gene under the conditions previously described, using primers for *Cyclin D2*, *RAR*-β-P2 promoter, and *Twist* described in (10, 14) and primers for *RASSF1A* and *HIN-1* described in (10). For each reaction, 1 μl of sodium bisulfite-treated DNA was added to 24 μl of reaction buffer [1.25 mM dNTP, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO, and 1.25 units RedTaq (Sigma, St. Louis, MO)] containing 100 ng each of forward and reverse primers specific to the unmethylated and methylated DNA sequences. Conditions for amplification of DNA were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension cycle of 72°C for 5 min.

Table 1 Distribution of risk factors across patients with primary breast cancer and lymph node metastasis

Risk factor	*n* (%)
Age >50	16 (64)
Age ≤50	9 (36)
Grade 2[a]	7 (33)
Grade 3	14 (67)
ER−/PR−[b]	17 (68)
ER+/PR+	8 (32)
Stage 2[a]	6 (30)
Stage 3	14 (70)

[a] Grade and stage not available for grade/stage four and grade/stage five samples, respectively.

[b] ER, estrogen receptor; PR, progesterone receptor.

Statistical Analysis. To assess the association between risk factors (age, tumor grade, stage, and estrogen receptor/progesterone receptor (ER/PR) status) and methylation of samples, proportions were compared. Significance was determined by Fisher's exact test. The association between tumor type and methylation was also assessed by Fisher's exact test. For comparison of methylation in paired samples from primary breast tumors and from LN metastasis, odds ratio were calculated and evaluated using McNemar's test. All 95% confidence intervals are calculated using exact methods.

Laser Capture Microdissection, RNA Isolation, and Reverse Transcription-PCR. Cryosections (5–8-μm) of LN metastasis samples (n = 7) were immediately fixed in 70% ethanol for 30 s, briefly stained with H&E before laser capture microdissection of malignant epithelial cells using a PixCell II laser capture microdissection system (Arcturus Engineering, Mountain View, CA) as per manufacturer's instructions. RNA from microdissected samples was isolated using RNeasy Mini kit (Qiagen Inc., Valencia, CA) and was reverse transcribed to cDNA using Superscript II (Invitrogen). Primers sequences for PCR were as follows: RAR-β2: CTTCCTGCATGCTCCAGGA (sense), CGCTGACCCCATAGTGGTA (antisense); Cyclin D2: CATGGAGCTGTGCCACG (sense), GTCCAGGTAATTCATGGCC (antisense) RASSF1A: GGCGTCGTGCGCAAAGGCC (sense), GGGTGGCTTCTTGCTGGAGGG (antisense) Twist: AGTCTCGCCGGCCGACGACA (sense), CGCGCTGCGCCTGCTGCTG (antisense); HIN-1: GGCCCTGAAGGCCCTGCTG (sense), TTTTGCTCTTAACCACGTTTATTGA (antisense);

Table 2 Prevalence of methylated *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* in breast cancer metastasis
Prevalence is compared in bone, brain and lung metastasis to prevalence in primary breast cancer using Fisher's exact test.

Tissue	n^a	*Cyclin D2* (%)	*P*	*RAR*-β (%)	*P*	*Twist* (%)	*P*	*RASSF1A* (%)	*P*	*HIN-1* (%)	*P*
Primary breast cancer	25	10/25 (40)		9/25 (36)		8/25 (32)		14/25 (56)		10/25 (40)	
Bone	12	6/12 (50)	0.73	9/10 (90)	<0.01	5/9 (56)	0.25	7/9 (78)	0.42	9/10 (90)	<0.01
Brain	8	7/8 (88)	0.04	6/7 (86)	0.03	5/7 (71)	0.09	4/6 (67)	0.99	7/7 (100)	<0.01
Lung	7	6/9 (67)	0.25	7/9 (78)	0.05	6/9 (67)	0.12	10/10 (100)	0.01	8/8 (100)	<0.01

[a] *n*, number of cases.

Table 3 Differential methylation in primary breast and its paired lymph node metastasis

Odds ratios (ORs) less than one indicate that lymph nodes (LNs) tend to have higher prevalence of methylation than the primary tumor. ORs are determined by McNemar's test and 95% confidence intervals (CI) are calculated using exact method.

	Primary +, LN −[a]	Primary −, LN +[a]	Matched OR	95% CI	*P*
Cyclin D2	0	1	0	0, 39	1.00
RAR-β	1	1	1	0.01, 78.5	1.00
Twist	1	4	0.25	0.005, 2.53	0.38
RASSF1A	1	2	0.50	0.008, 9.60	1.00
HIN-1	1	8	0.125	0.003, 0.93	0.04

[a] Number of samples methylated (+) or unmethylated (−) in primary breast carcinoma and its paired lymph node metastasis.

36B4: GATTGGCTACCCAACTGTTGCA (sense), CAGGGGCAGCAGCCACAAAGGC (antisense). For PCR, 1 μl of cDNA was used in a 25-μl reaction volume, which for RAR-β, Cyclin D2, RASSF1A, Twist, and 36B4, contained 12.5 μl of 2× PCR mix (Life Technologies, Inc.), 200 nM each primer, and 1.25 units of RedTaq (Sigma, St. Louis, MO); and for HIN-1, 24 μl of reaction buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO, 1.25 mM dNTP, and 1.25 units RedTaq, and 400 nM each primer]. A touch down PCR was used for HIN-1, RASSF1A. and Twist: 95°C for 3 min, 5 cycles of 95°C for 30 s and 68°C for 2 min, 5 cycles of 95°C for 30 s, 64°C for 1 min, and 70°C for 1 min, then 35 cycles of 95°C for 30 s, 61°C for 1 min, and 70°C for 1 min, followed by 70°C for 5 min. RAR-β and Cyclin D2 were amplified by regular PCR for 40 cycles at 58°C and 60°C annealing temperatures, respectively. 36B4 was amplified for 35 cycles at 56°C annealing temperature.

***In Situ* Hybridization for HIN-1.** Paraffin-embedded sections were deparaffinized and processed as described by Porter *et al.* (19). Six primary tumors, six LN-metastases, and five samples of each of the distant metastases to bone, brain, and lung were tested. The slides were scored for intensity of staining after examining at least 10 fields in each slide.

RESULTS AND DISCUSSION

Frequent Incidence of Hypermethylated Genes in Distant Metastasis. Sodium bisulfite-treated DNA from 8 to 12 samples each from breast cancer metastasis to the bone, brain, and lung were evaluated by methylation-specific PCR (MSP) for the presence of hypermethylated promoter sequences in the *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* genes. These genes were selected for analysis because normal breast epithelium, stroma, as well as peripheral WBCs are most often negative for methylation (9, 10, 14, 17). Evaluable results were obtained from 7 to 12 samples in each group. The incidence of hypermethylated genes in the three distant organ-metastases varied between 50 and 100% (Table 2). A representative MSP analysis is shown in Fig. 1.

To determine whether there was a significant increase in the frequency of hypermethylation of the five genes in distant metastases compared with their primary site, we analyzed the DNA from 25 LN-positive primary breast carcinomas (Tables 1 and 2). Compared with the LN-positive primary breast carcinomas, there was a statistically significant increase in the incidence of hypermethylation in *RAR*-β and *HIN-1* genes in lung, brain, and bone metastasis ($P < 0.01$). An increased incidence (67–100%) of methylation in *Cyclin D2*, *Twist*, and *RASSF1A* was also observed; however, the differences were statistically significant for only *Cyclin D2* ($P = 0.04$) in the brain, and *RASSF1A* ($P = 0.01$) in the lung metastases (Table 2).

We next asked whether hypermethylation in these five genes correlates uniquely only with the ability of tumor cells to metastasize to distant organs, or whether it is a change occurring in local metastasis as well. To address this question, DNAs from 25 primary tumors and LN metastases from the same patients were also examined by MSP (Fig. 1). When analyzing differential methylation in paired samples, we observed a trend of higher prevalence of methylation for all five genes in LN metastasis than in the primary tumor, reaching statistical significance ($P = 0.04$) for only *HIN-1* (Table 3).

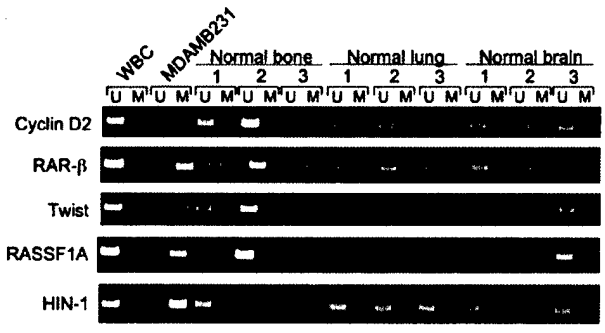

Fig. 2 Incidence of hypermethylation in normal bone, lung, and brain tissue. Methylation-specific PCR (MSP) analysis was performed using primers specifically amplifying *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* gene promoters. Peripheral WBCs (*WBC*) and MDA-MB231 cells served as controls for unmethylated (*U*) and hypermethylated (*M*) genes, respectively. PCR products were visualized using ethidium bromide after electrophoresis on a 2% agarose gel.

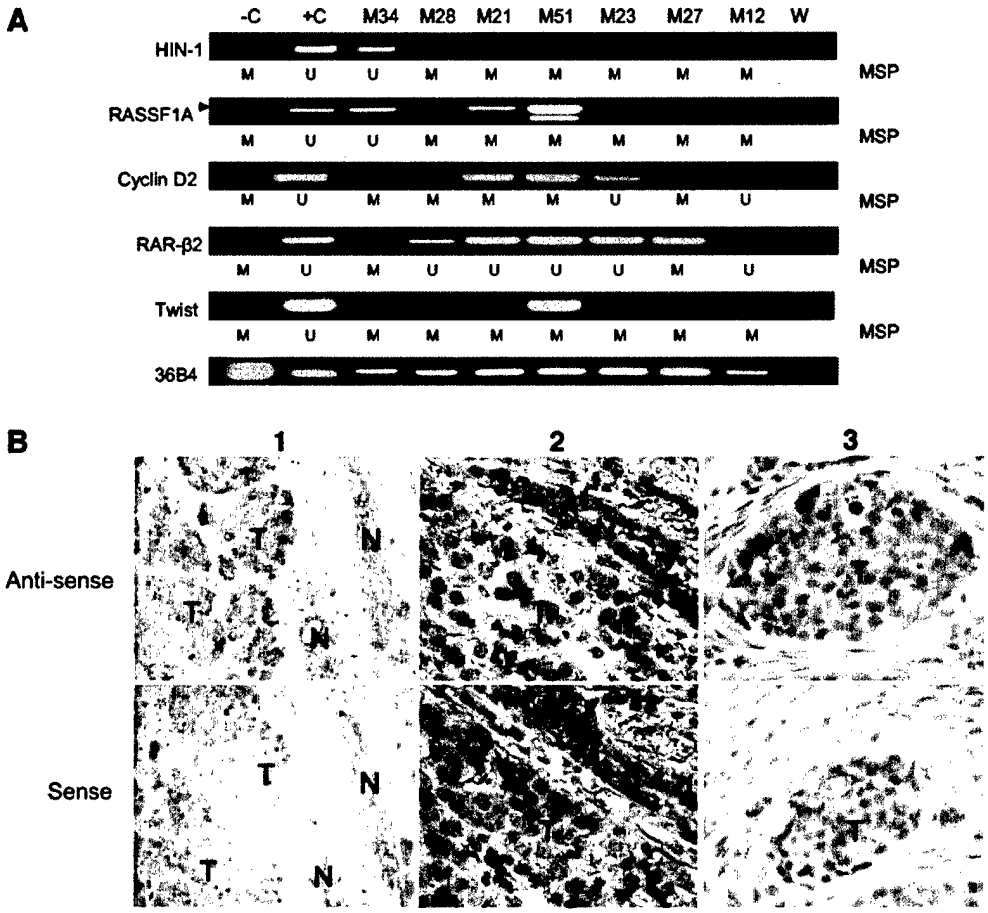

Fig. 3 Correlation of hypermethylation of genes with expression of their mRNA: *A*, reverse transcription-PCR analysis of expression of HIN-1, RASSF1A, Cyclin D2, RAR-β2, Twist, and 36B4 (a ribosomal protein gene) in microdissected epithelial cells from lymph node (LN) metastasis (*M34, M28, M21, M51, M23, M27, M12*). The breast cancer cell line, MDA-MB231, which is hypermethylated for all five genes and lacks detectable expression, served as a negative control (*-C,*) and a primary breast carcinoma sample with unmethylated DNA that shows expression for all five genes served as a positive control (*+C*). Water (*W*) was a no cDNA control. *36B4* served as a positive control for the presence of cDNA in each of the samples. Two bands are coamplified with the primer set for RASSF1 cDNA, RASSF1A (*top band*) and RASSF1F (*bottom band*). Methylation status of each gene, determined by methylation specific PCR (*MSP*), is reported below each sample for direct comparison as methylated (*M*) or unmethylated (*U*). *B, in situ* hybridization (ISH) analysis for expression of HIN-1 mRNA; *T,* tumor; *N,* normal. ISH was performed on paraffin-embedded sections of human primary breast tumor tissue (1), metastasis to the lung (2), and metastasis to the bone (3) using HIN-1 sense and antisense digoxigenin-labeled riboprobes. Sections were counterstained with hematoxylin and were visualized by light microscopy. *Panel 1, ×200; Panels 2 and 3, ×400.*

We also determined the association of risk factors, *i.e.,* age, tumor grade, stage, and ER/PR status with methylation status of five genes in either primary breast tumor or its paired LN metastasis. No significant association of risk factors with methylation status of the five genes was observed except for ER+/PR+ status, which, in the primary breast tumor, was significantly associated with *HIN-1* methylation ($P = 0.03$; data not shown).

Hypermethylated Genes Are Not Detectable in Adjacent Normal Host Tissue. Our previous investigations using MSP analysis have confirmed the absence, in general, of hypermethylation of these five genes in normal breast samples and in peripheral WBCs (9, 10, 14). However, one could argue that the high frequency of hypermethylation in distant metastasis is a characteristic of the normal host cells rather than the metastasis. To resolve this question, we investigated DNA from normal tissues of the site of origin of the distant breast metastases: normal bone, brain, and lung. MSP was performed on histopathologically normal tissues obtained at autopsy, or other surgical procedures for noncancer-related diseases. DNA from 6 to 10 samples from each site was analyzed by MSP. All of the DNAs were negative for the presence of hypermethylated gene sequences (representative data in Fig. 2). Thus, the adjacent normal tissues are most likely not the source of the frequent hypermethylation observed in distant metastasis. However, without performing MSP analysis on microdissected carcinoma cells and the adjacent stroma, we cannot exclude the possibility that the host cells immediately adjacent to the metastatic lesion contain hypermethylated genes, and that they are influenced by the epigenetic status of DNA and histone levels in metastatic cells.

In summary, hypermethylated genes were more frequently found in the local and distant metastasis compared with the primary breast carcinomas. Adjacent normal tissue from the host site may not be the source of the hypermethylation observed in these tissues. These results suggest that the increase in frequency of hypermethylated genes in distant metastasis may be an important event in the progression of breast cancer. Such a marked increase in the frequency of promoter hypermethylation also suggests that these losses may confer survival advantage to the disseminated cells at the distant site.

Correlation of Hypermethylation of Genes with Expression of Their mRNA. Dysfunctional epigenetic control by aberrant methylation of DNA is now well accepted as an important mechanism for shutting down gene expression and leading to loss of tumor/growth suppressor function. We sought to determine whether hypermethylation resulted in the loss of gene expression, as has been previously shown for Cyclin D2, RAR-β2, RASSF1A, and HIN-1 (9, 11, 15, 17). We performed reverse transcription-PCR to test the expression of all five genes on RNA extracted from microdissected epithelial cells from seven LN metastasis samples. MDA-MB231 cells served as control for DNA that is hypermethylated for all five genes, and shows loss of expression of their mRNA, and a primary carcinoma sample as a control that shows unmethylated DNA and the presence of detectable mRNA (Fig. 3*A*). With few exceptions (8 of 35), expression of the mRNA correlated with hypermethylation of the promoter sequences. Lack of perfect correlation in these cases is not unexpected, because control of gene expression is a complex process, in which the chromatin conformation, availability of cofactors, and repressor and enhancer molecules all play a part, and methylation status alone does not determine gene expression. Our data show that methylation is one of the important determinants, because in 77% of the cases, expression of the five genes in microdissected breast carcinoma metastasis in the LNs correlates with hypermethylation of the promoter sequences.

To visualize the expression of HIN-1 at the cellular level, we performed mRNA *in situ* hybridization for HIN-1 expression in the primary tumor and distant metastasis. HIN-1 expression was lost in four of six tumors with methylated *HIN-1* genes. Although HIN-1 is expressed in the primary tumor shown in Fig. 3*B*, there is complete loss of expression in its LN metastasis (not shown) and in each of the distant metastases to the lung, bone (Fig. 3*B*), and brain (not shown). Here again, for HIN-1, there is a fairly direct correlation between promoter hypermethylation and loss of mRNA expression. Loss of the cytokine HIN-1, which occurs very early and very frequently in breast tumorigenesis, could have profound biological effects that are yet to be elucidated.

In conclusion, we show for the first time, the methylation patterns of *Cyclin D2*, *RAR-β*, *Twist*, *RASSF1A*, and *HIN-1* genes in distant metastasis to common sites of breast cancer dissemination: the LNs, bone, brain, and lung. A high frequency of hypermethylated genes was revealed in locoregional and distant metastasis compared with primary breast carcinoma. This report provides information that may be important to the biology of distant metastasis and that may assist in the design of therapeutic modalities. It is already recognized that methylated genes could provide unique targets for therapy (20). Re-expression of these genes, in particular RAR-β genes, can lead to growth inhibition and death of cancer cells in the presence of retinoic acid (RA). In particular, RA-induced RAR-β gene expression in cells is mediated through a RA response element (β-RARE; Ref. 21). As already shown (22), histone deacetylase inhibitors appear to relieve the repressive chromatin conformation at the β-RARE-containing promoter of *RAR-β* and restore RA responsiveness. Endogenous up-regulation of RAR-β by RA is very important because it plays a critical role in amplifying the RA response, and is required for RA-induced growth inhibition and apoptosis in breast cancer. This was demonstrated by treating human breast cancer cells and xenografts carrying a methylated *RAR-β* promoter with a combination of the re-acetylating agent Trichostatin A and RA, which resulted in a significant increase in the degree of apoptosis and tumor-growth inhibition as compared with treatment with either agent alone (22). On the basis of preclinical studies, it is tempting to speculate that differentiation therapy for distant metastasis could be improved through reversing the silenced state of *RAR-β* by using a combination of chromatin remodeling agents and retinoids. Many genes, including each of the five tested in this study, can be reactivated in metastatic breast cancer cell lines using demethylating agents, histone deacetylase inhibitors, or a combination of the two.[6] Our molecular data provide reinforcement for studies aimed at the design and development of therapeutic strategies targeting specific epigenetic changes in metastatic breast cancer.

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Appendix 4

Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer

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AQ: A

ABSTRACT

If detected early, breast cancer is eminently curable. To detect breast cancer in samples with little cellularity, a high level of sensitivity is needed. Tumor-specific promoter hypermethylation has provided such a valuable tool for detection of cancer cells in biological samples. To accurately assess promoter hypermethylation for many genes simultaneously in small samples, we developed a novel method, quantitative multiplex-methylation-specific PCR (QM-MSP). QM-MSP is highly sensitive (1 in 10^4–10^5 copies of DNA) and linear over 5 orders of magnitude. For *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*, we observed significant differences in both the degree ($P < 0.003$) and incidence ($P < 0.02$) of hypermethylation between normal and malignant breast tissues. Evaluation of the cumulative hypermethylation of the four genes within each sample revealed a high level of sensitivity (84%) and specificity (89%) of detection of methylation. We demonstrate the application of this technique for detecting hypermethylated *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* in 50–1000 epithelial cells collected from breast ducts during endoscopy or by lavage. Such an approach could be used in a variety of small samples derived from different tissues, with these or different biomarkers to enhance detection of malignancy.

INTRODUCTION

Epigenetic alterations including hypermethylation of gene promoters are proving to be consistent and early events in neoplastic progression (1–4). Such alterations are thought to contribute to the neoplastic process by transcriptional silencing of tumor suppressor gene expression and by increasing the rate of genetic mutation (5, 6). DNA methylation is reversible because it does not alter the DNA sequence; however, it is heritable from cell to cell. Methylated genes can serve as biomarkers for early detection of cancer. Widschwendter and Jones (4) reviewed >40 genes whose expression is lost in breast cancer because of promoter hypermethylation, and we and others have studied hypermethylation of genes, including *NES-1* (7–10), *APC* (11–13), *Cyclin D2* (14, 15), *RARB* (16–18), *TWIST* (19), *RASSF1A* (15, 20, 21), and *HIN1* (22) in tissue (23) and ductal lavage fluids (19). Multigene promoter hypermethylation occurs commonly in tumors (24, 25). Because methylation changes often appear early in disease, detection of hypermethylated genes could identify tissues derived from patients with increased risk. Furthermore, the reversible nature of methylation offers the potential to revert aspects of the cancer phenotype with the appropriate therapy (26).

Tumor DNA can be found in various body fluids, and these fluids can potentially serve as diagnostic material (19, 27–29). Evaluation of tumor DNA in these fluids requires methods that are specific as well as sensitive. For example, a PCR-based technique called methylation-specific PCR (MSP) can detect 1 copy of methylated DNA in 1000 unmethylated copies of genomic DNA (30). Palmisano *et al.* (31) and others (32, 33) have modified this approach to coamplify several genes simultaneously in a nested or multiplex MSP assay. This method has been used to establish the frequency of gene promoter hypermethylation among patients with pulmonary (31) and esophageal (33) carcinoma. However, the method cannot quantitatively measure the levels of gene methylation because the read-out is gel-based and qualitative ("all or nothing"), based on the visual detection of the presence or absence of a band on a gel. The issue gains importance because benign tissues often show low levels of methylation in several genes.

AQ: B

To evaluate the degree of gene methylation within a single sample, quantitative MSP (Q-MSP) methods have been developed (15, 34–39). High and low levels of methylation may help to stratify different types or stages of carcinoma. The Q-MSP method is based on real-time PCR that uses fluorogenic probes to increase the assay specificity and the sensitivity; Q-MSP can detect one copy of the methylated marker gene among 10,000 unmethylated copies (36). The addition of a fluorogenic probe makes the technique more informative, quantitative, and suitable for clinical format. This technique is now becoming widely used (15, 34–39). However, analyses of multiple genes require additional quantities of template DNA. A dilemma is how best to distribute the available DNA to allow quantitative analyses of many different genes from precious small samples.

We have developed a technique called quantitative multiplex-MSP (QM-MSP) to coamplify many genes from quantities of sample previously used for just one gene. This technique combines multiplex PCR and Q-MSP in such a way that a panel of five genes can be coamplified in tissues derived from different sources, including those from ductal lavage, endoscopy, and fine-needle aspirates, in which the amount of DNA is limiting, as well as in larger samples, such as formalin-fixed, paraffin-embedded sections of core biopsies. This technique can be used to define the extent of gene promoter hypermethylation in normal tissues on a gene-by-gene basis and provides the ability to discriminate between normal/benign and malignant tissues.

MATERIALS AND METHODS

Tissues and Cells. Paired primary invasive breast carcinomas and adjacent normal tissues (frozen tissue), paraffin-embedded normal breast (routine reduction mammoplasty), and primary breast carcinoma sections were obtained from the Surgical Pathology archives of The Johns Hopkins Hospital following approval by the Institutional Review Board. The percentage of epithelial cells in each tissue section ranged from 30 to 80% on each slide. Ductal cell samples obtained by lavage of high-risk women and endoscopy of women with biopsy-proven breast cancer before surgery were provided after cytological diagnosis. Histopathological diagnosis of the resected specimen from patients undergoing endoscopy was obtained from Surgical Pathology. Human sperm was obtained from a healthy volunteer. The MDA-MB231 (231) breast cancer cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured

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Table 1 *Sequences of multiplex primers used in round 1 of PCR*

Primer name	Primer sequence (5′ → 3′)	Orientation
Cyclin D2 Ext F	tatttttttgtaaagatagttttgat	Forward
Cyclin D2 Ext R	tacaactttctaaaaaataaccc	Reverse
RASSF1A Ext F(2)	gttttatagttttttgtatttagg	Forward
RASSF1A Ext R(2)	aactcaataaactcaaactccc	Reverse
TWIST Ext R(4)	cctcccaaaccattcaaaaac	Forward
TWIST Ext F(3)	gagatgagatattatttattgtg	Reverse
RARB Ext F	gtaggagggtttatttttttgtt	Forward
RARB Ext R(2)	aattacattttccaaacttactc	Reverse
HIN1 Ext F(2)	gtttgttaagaggaagtttt	Forward
HIN1 Ext RSEQ	ccgaaacatacaaaacaaaaccac	Reverse
ACTB Ext F	tatataggttggggaagtttg	Forward
ACTB Ext R	tataaaaacataaaacctataacc	Reverse
ACTB Ext F	tatataggttggggaagtttg	Forward

as directed. Peripheral blood leukocytes were isolated from blood collected from normal volunteers after receipt of informed consent following review by the Johns Hopkins Institutional Review Board.

DNA Extraction from Tissues and Peripheral Blood Cells. For each tissue, the lesion was confirmed on a H&E-stained section. For DNA extraction, one 5-μm tissue section from the same block was deparaffinized in xylene (20 min), scraped from the slide, and extracted in 100 μl of buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.5% SDS] containing 40 μg proteinase K for 16 h at 50°C. For extraction of DNA from ductal cells, the number of cells on each Papanicolaou- or Diff-Quick-stained (American Scientific Products, McGraw, IL) cytospin preparation was counted, the coverslip was removed by treatment with xylene, and cells were scraped and transferred to 50 μl of buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.5% SDS] containing 40 μg/ml proteinase K and 200 ng of salmon sperm carrier DNA. After proteinase K treatment, samples were heat-inactivated at 70°C for 10 min and centrifuged at 16000 × *g* for 10 min. Fifty μl of the supernatant were used directly as a source of DNA for sodium bisulfite treatment.

For leukocytes, frozen tissues, and 231 cells, DNA was extracted with phenol–chloroform (40). Human sperm DNA (HSD) was isolated by use of the PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) and stored at 4°C. One μg of purified DNA was modified by sodium bisulfite treatment.

Sodium Bisulfite Treatment of DNA. Tissue, control and cell line DNAs were treated with sodium bisulfite and analyzed by MSP as described by Herman *et al.* (30). This process converts nonmethylated cytosine residues to uracil, whereas methylated cytosines remain unchanged. Bisulfite-modified samples were aliquoted and stored at −80°C.

Probes and Primers. The sequences of primers used for multiplex and for amplifying unmethylated and methylated CpG islands by Q-MSP are shown in Tables 1 and 2. Gene-specific probes were obtained from Applied Biosystems (Foster City, CA), and primers were obtained from Invitrogen Corporation (Carlsbad, CA). For methylated *Cyclin D2* and *RASSF1A* genes, the Q-MSP primers and probes were as described in Lehmann *et al.* (15). Methylation-independent Q-MSP primers and probes for β-actin (*ACTB*) were as described by Eads *et al.* (36). All other sequences for methylation-dependent primers were designed in known regions of promoter hypermethylation in breast carcinoma; each Q-MSP primer set (forward, reverse, and probe) contained 7–12 CpG dinucleotides of the promoter sequence and numerous independent cytosine residues. T1,T2,AQ:

QM-MSP. The QM-MSP procedure required two sequential PCR reactions (Fig. 1). In the first PCR reaction (the multiplex step), 1 μl of sodium bisulfite-treated DNA was added to 24 μl of reaction buffer [1.25 mM deoxynucleotide triphosphates, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO, and 2.5–5 units of Platinum *Taq* (Invitrogen)] containing 100 ng each of the forward and reverse primers. Conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension cycle of 72°C for 5 min. The PCR products were diluted up to 125 μl with water and stored at −20°C. For this reaction, the input DNA ranged from 50 ng (purified DNA) to ~40 pg (for some ductal cell samples). F1

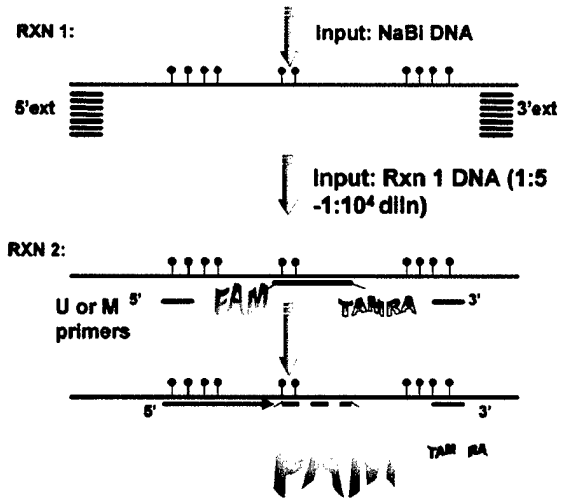
For the second round (the Q-MSP step), 1 μl of the diluted PCR product from reaction 1 was used directly or after further dilution of up to $1{:}10^4$ (when 50 ng DNA was used in reaction 1). The diluted DNA was added to the Q-MSP reaction buffer containing 16.6 mM $(NH_4)_2S0_4$, 67.0 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10.0 mM β-mercaptoethanol, 0.1% DMSO, 200 μM deoxynucleotide

Table 2 *Sequences of Q-MSP[a] primers used in round 2 PCR*

Primer name	Q-MSP primer sequence (5′ → 3′)	Orientation	Status
Cyclin D2 RT-FM[b]	tttgatttaaggatgcgttagagtacg	Forward	M
Cyclin D2 RT-RM[b]	actttctccctaaaaaccgactacg	Reverse	M
Cyclin D2 M Probe[b]	6FAM-aatccgccaacacgatcgacccta-TAMRA	Reverse	M
Cyclin D2 RT-FUM	ttaaggatgtgttagagtatgtg	Forward	U
Cyclin D2 RT-RUM	aaactttctccctaaaaaccaactacaat	Reverse	U
Cyclin D2 UM Probe	6FAM-aatccaccaacacaatcaaccctaac-TAMRA	Reverse	U
RASSF1A RT-FM[b]	gcgttgaagtcggggttc	Forward	M
RASSF1A RT-RM[b]	cccgtacttcgctaactttaaacg	Reverse	M
RASSF1A M Probe[b]	6FAM-acaaacgcgaaccgaacgaaacca-TAMRA	Reverse	M
RASSF1A RT-FUM	ggtgttgaagttggggtttg	Forward	U
RASSF1A RT-RUM	cccatacttcactaactttaaac	Reverse	U
RASSF1A UM Probe	6FAM-ctaacaaacacaaaccaaacaaaacca-TAMRA	Reverse	U
TWIST RT-FM	gttagggttcgggggcgttgtt	Forward	M
TWIST RT-RM	ccgtcgccttcctccgacgaa	Reverse	M
TWIST M-Probe	6FAM-aaacgatttccttcccgccgaaa-TAMRA	Reverse	M
TWIST RT-FUM (3)	ggtttgggggtgttgtttgtatg	Forward	U
TWIST-RT-RUM (3)	cccacctcctaaccaccctcc	Reverse	U
TWIST UM Probe	6FAM-aaacaatttccttccccaccaaaaca-TAMRA	Reverse	U
RARB RT-FM	agaacgcgagcgattcgagtag	Forward	M
RARB RT-RM	tacaaaaaacctccgaatacgtt	Reverse	M
RARB M Probe	6FAM-atcctaccccgacgatacccaaac-TAMRA	Reverse	M
RARB RT-FUM	ttgagaatgtgagtgatttgagtag	Forward	U
RARB RT-RUM	ttacaaaaaaccttccaaatacattc	Reverse	U
RARB UM Probe	6FAM-aaatcctaccccaacaatacccaaac-TAMRA	Reverse	U
HIN1 RT-FM	tagggaaggggtacgggttt	Forward	M
HIN1 RT-RM	cgctcacgaccgtaccctaa	Reverse	M
HIN1 M Probe	6FAM-acttcctactacgaccgacgaacc-TAMRA	Reverse	M
HIN1-RT-FUM (2)	aagtttttgaggt ttgggtaggga	Forward	U
HIN1 RT-RUM (2)	accaacctcacccacactccta	Reverse	U
HIN1UM Probe	6FAM-caacttcctactacaaccaacaaacc-TAMRA	Reverse	U
ACTB F	tggtgatggaggaggtttagtaagt	Forward	Indep
ACTB R	aaccaataaaacctactcctcccttaa	Reverse	Indep
ACTB Probe	6FAM-accaccaccaacacacaataacaaacaca-TAMRA	Reverse	Indep

[a] Q-MSP, quantitative methylation-specific PCR; M, methylated; 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; U, unmethylated; Indep, methylation-independent.

[b] ●●●.



AQ: K Fig. 1. Scheme for the quantitative multiplex methylation-specific PCR. In reaction 1 (*RXN 1*), a cocktail of gene-specific primer pairs is used to coamplify DNA for multiple genes independent of their DNA methylation status. In reaction 2 (*RXN 2*) quantitative real-time PCR is performed with gene-specific primers, using the DNA template derived from the end product of RXN 1 (diluted 1:5–1:10^4). DNA was analyzed in separate wells, using methylation status-specific [*U*, unmethylated; *M*, methylated) primers (forward, reverse, and probe, conjugated with the 6-carboxyfluorescein (*FAM*) label and 6-carboxytetramethylrhodamine (*TAMRA*) quencher]. The oligonucleotide probe is progressively degraded with each cycle of PCR by the 5′–3′ nuclease activity of the DNA polymerase; therefore, the fluorescence signal generated by 6-carboxyfluorescein is directly proportional to the extent of DNA amplification. % M = 100 × [no. of copies of M/no. of copies of (unmethylated + methylated)], as determined by the absolute quantification method computed against a standard curve. *NaBi*, sodium bisulfite; *ext*, extension; *diln*, dilution.

triphosphates, 1.25 units of Platinum DNA *Taq* Polymerase (Invitrogen), and 1× ROX (Invitrogen) in a final volume of 25 μl. Six hundred nM each of two primers (forward and reverse) and 200 nM labeled probe (Applied Biosystems) were also present. The reaction was carried out in a 96-well reaction plate in an ABI Prism 7900HT Sequence Detector (Applied Biosystems). The reaction conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 15 s and 60–65°C (depending on the primer set) for 1 min, with a 10-min extension at 72°C. For each gene included in the reaction plate, the following were used to create standard curves and to provide controls: (*a*) serially diluted stock multiplexed HSD/231 DNA (described below, to establish a standard curve); (*b*) 40,000 copy (40 K) standards; (*c*) no-template control; and (*d*) a known DNA ("1% M" control) to ensure consistency among runs. In addition, 100% methylated DNA (231 cell DNA), 0% methylated DNA (HSD), and a sample lacking template DNA from the first PCR reaction (diluted 1:5) were present as controls. All of the above samples were analyzed with primer sets for both methylated and unmethylated DNA. Because the 1% M control values have remained constant over a period of 1 year (data not shown) we conclude that the assay is stable and reproducible.

Preparation of Standards. A stock of multiplexed DNA was prepared as follows: PCR was performed in a reaction that contained all first-round gene primer pairs (Table 1) as well as a mixture of 50 ng each of sodium bisulfite-treated genomic 231 and HSD. Serial dilutions of this stock DNA were used to establish a standard curve in the real-time PCR reaction. To do this, the cycle threshold (C_T; the cycle in which the signal exceeds the background) of each dilution was determined during the Q-MSP reaction and then plotted against the dilution to generate a line for the standard curve. For each reaction plate, the standards were diluted from the same stock stored frozen at −80°C for all assays, and new dilutions were made each time. All assays had a correlation coefficient of the standard curve of 0.99 or higher and a slope of approximately −3.33, indicating 2-fold increases in PCR product per cycle in the linear phase of the quantitative PCR reaction.

Copy Number Standard. For the preparation of this standard, unmethylated or methylated genomic DNAs were amplified for each gene separately by use of a gene-specific pair of external primers and 50 ng of sodium bisulfite-treated genomic DNA derived from either MDA-MB231 (100% methylated) or HSD (100% unmethylated). A single band was observed by gel electrophoresis. The reaction products were then purified with the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA) and eluted in 100 μl of water. The eluate was quantitated by use of a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE), and the DNA concentration (μg/μl) was determined (A_{260}). The molecular weight (μg/μmol) of the PCR product was AQ: D calculated by use of Biopolymer Calculator v4.1.1 (C. R. Palmer).[5] The AQ: E concentration of each gene template control was adjusted to 3×10^{10} copies.μl in 1 mg/ml salmon sperm carrier DNA, and then a cocktail of unmethylated and methylated template control was immediately prepared that contained 4×10^6 copies/μl each of the genes in 1 mg/ml salmon sperm DNA. This stock was stored at −80°C. For each reaction plate the stock was diluted 100-fold to 40,000 copies/well. We used this known quantity of standard (40,000 copies/well, denoted "40K" control), prepared as described above, to transform the standard curve to represent copy number. To accomplish this the C_T of the 40 K control was determined during the Q-MSP reaction and plotted on the line obtained for the standard curve. The copy number for each dilution was then "back calculated," based on where the 40K C_T intersected the standard curve. Sample 40K had approximately equal amounts of unmethylated and methylated DNA for each of the five genes (*RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB*) along with carrier salmon sperm DNA (10 μg/ml).

Calculation of Percentage of Methylation. The relative amount of methylation in each unknown sample was calculated as % M = 100 × [no. of copies of methylated DNA/(no. of copies of methylated + unmethylated DNA)]. The sum of unmethylated plus methylated DNA (U + M) was used as an approximation of the total number of copies present of a target gene. To determine the number of copies of methylated and unmethylated DNA, we mixed sample DNA with Q-MSP reaction buffer after the multiplex reaction, assayed the mixture with methylated primers and unmethylated primers (in separate wells) in the Q-MSP reaction, and then determined the C_T was for each. Using the ABI Prism SDS 2.0 software supplied by Applied Biosystems (Foster City, CA) with the 7900 HT Sequence Detector, we extrapolated the number of copies of methylated and unmethylated DNA from the respective standard curves, using the sample C_T and applying the absolute quantification method according to the manufacturer's directions. Only values falling within the range covered by the standard curve (usually 100–10,000,000 copies) were accepted.

Direct Q-MSP of Genomic DNA. For direct Q-MSP, standard curves were prepared using 10 pg, 100 pg, 1 ng, and 10 ng total genomic 231 DNA (fully methylated) or HSD (fully unmethylated), according to the absolute quantitation method described by Applied Biosystems in the 7900 HT Sequence Detector manual. The concentrations of methylated and unmethylated DNA were extrapolated from these curves, and the percentage of methylation was calculated as % M = 100 × [ng methylated gene A/(ng methylated gene A+ unmethylated gene A)], where total target gene DNA was taken as the sum of U + M. For purposes of comparing these results with methods that use β-actin (*ACTB*) as a reference DNA, we also computed the percentage of methylation using two other formulas. We calculated % M = 100 × (ng methylated gene A in tumor/ng *ACTB* gene in tumor), or essentially as described by Trinh *et al.* (37), who calculated the percentage of methylation as % M = 100 × [(ng methylated gene A in tumor/ng *ACTB* in tumor)/(ng methylated gene A in 231 controls/ng *ACTB* in 231 controls)].

Statistical Analysis and Graphical Representation of Data. Statistical analyses and plotting of data were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA) and Stata 7.0 (Stata Corporation, College Station, TX). *P* values < 0.05 were considered significant, and all tests were two-tailed. The nonparametric Mann–Whitney test was used to test whether the samples were from identical distributions, indicating that their medians were equal. Sample means were compared by use of the unpaired *t* test, assuming unequal variances (Welch's correction). For testing of means, data were transformed as a function of $Ln_e(\%M + 1)$ where stated to fulfill the assumption of normality. The Fisher's exact test was used to test whether the differences between the incidence of positivity for methylation in tumor and nontumor samples were significant.

[5] http://paris.chem.yale.edu/extinct.html.

RESULTS

Validation of the Two-Step QM-MSP Assay. Multiplex PCR was accomplished by performing two sequential PCR reactions (Fig. 1). In the first PCR reaction, a cocktail of gene-specific primers was used to coamplify *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* (Table 1). These external primer pairs were complementary to the sequences flanking the CpG island that was to be assayed in the second PCR reaction. External primers were selected to exclude CG dinucleotides, thereby rendering DNA amplification independent of the methylation status. In the second reaction, quantitative analysis of methylated and unmethylated DNA for each gene was performed separately with the primer pairs and probes shown in Table 2.

Unmethylated and Methylated Primers are Equally Efficient in Amplifying Sodium Bisulfite-Converted DNA. Primer sets for specifically amplifying methylated (M) or unmethylated (U) DNA (Table 2) were designed for comparable performance; to confirm this we plotted, the ΔC_T (C_T M − C_T U) as a function of sample dilution over a wide range of dilutions (10^{-3}–10^{-8}) of the standard stock HSD/231 DNA. Analyses were performed as shown for *RASSF1A* (Fig. 2). The ΔC_T was approximately the same for all dilutions, as shown by the horizontal nature of the line, indicating that the primer sets were equally efficient over 5 logs of template quantities (Fig. 2*A*). In addition, for both unmethylated and methylated DNA, the slopes of the standard curves were approximately −3.33, which is reflective of a 2-fold increase in PCR product per cycle during the linear phase of real-time PCR. Finally, the correlation coefficient (R^2) of 0.999 provided evidence of linearity over the entire range of template concentration (Fig. 2*B*). Similar results were obtained for each of the other genes in this study (data not shown).

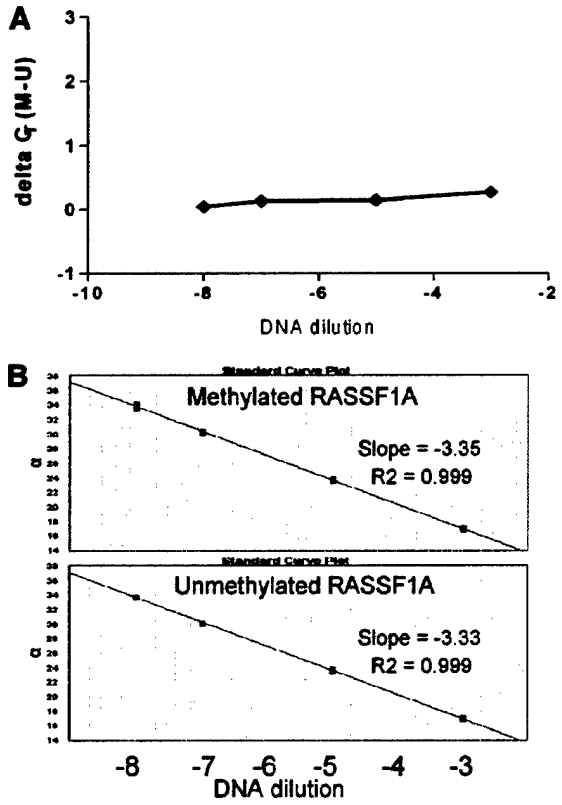


Fig. 2. Efficiency of quantitative multiplex methylation-specific PCR. For verification of the comparable function and efficient performance of the unmethylated (*U*) and methylated (*M*) primers, QM-MSP was performed using *RASSF1A* U and M primer sets to amplify serially diluted (10^{-3}–10^{-8}) fragments of multiplexed standard stock human sperm DNA/MDA-MB231 DNA. The cycle threshold (C_T) was determined for each dilution of DNA. *A*, plot of ΔC_T ($\Delta C_T = C_T$ M − C_T U) *versus* dilution. Nearly identical ΔC_T values for each DNA dilution indicate uniform primer performance over 5 logs. *B*, standard curve plots (C_T *versus* quantity) of serially diluted DNA. The slope of −3.33 reflects a 2-fold amplification of DNA per cycle (high efficiency). The correlation coefficient (R^2) of 0.999 shows the high degree of linearity over the entire range.

Table 3 *Determining sensitivity of quantitative multiplexed methylation-specific PCR*

	Methylation[a] (%)			
	RASSF1A	*TWIST*	*HIN1*	*Cyclin D2*
Copies M[b] and U template[c,d] (n)				
1000/100,000	0.9220	2.7604	4.8024	1.5760
100/100,000	0.0602	0.2482	0.6273	0.1525
10/100,000	0.0078	0.0272	0.0024	0.0025
1/100,000	0.0072	0.0012	0.0000	0.0000
0.1/100,000	0.0000	0.0000	0.0000	0.0000
HSD (100% U control)	0.0000	0.0003	0.0000	0.0000
231 (100% M control)	99.9981	100.0000	100.0000	100.0000
Water	0.0000	0.0000	0.0000	0.0000
Copies M and U[c,e] (n)				
300/29,700	0.7823	1.2190		
30/2,970	0.7195	0.9376		
3/297	0.2390	0.1451		

[a] The percentages of methylation of *RASSF1A*, *TWIST*, *HIN1*, and *Cyclin D2* from the quantitative multiplexed methylation-specific PCR assay are shown. The assay sensitivity is 1–10 in 100,000 for methylated DNA, and it detects as few as 1–3 copies of methylated DNA.

[b] M, methylated; U, unmethylated; HSD, human sperm DNA; 231, MDA-MB231.

[c] Purified methylated and unmethylated stock DNA template were mixed in the proportions indicated.

[d] Unmethylated DNA was kept constant as the amount of methylated DNA was decreased.

[e] A 1% M control was kept constant as the total quantity of DNA decreased.

Specificity and Sensitivity. To assess the sensitivity and specificity of the QM-MSP method, we performed a mixing experiment, using column-purified, PCR-amplified fragments of sodium bisulfite-modified DNA as template (Table 3). The amount of unmethylated DNA was kept constant (100,000 copies/well), and the amount of methylated DNA was decreased (1000–0.1 copy/well). Although the general efficiency of real-time PCR is known to fall off at <100 copies, it was expected that some level of methylation would be detected at concentrations <100 copies, although probably not in a linear manner. For *RASSF1A* and *TWIST*, methylation was detected at 1 methylated copy in 100,000 unmethylated copies, and for *Cyclin D2* and *HIN1*, methylation was detected at 10 in 100,000 copies. Therefore, the overall sensitivity of the method was 1–10 in 100,000.

The highly specific performance of the methylated primers was demonstrated by the use of HSD control (100% unmethylated DNA) and the 0.1/100,000 sample (diluted to <1 copy of methylated in the presence of 100,000 copies of unmethylated DNA per well), both of which showed 0% M in the Q-MSP reaction (Table 3; Fig. 3). Likewise, no unmethylated signal (0% U) was detected in 231 methylated DNA control (100% methylated DNA). It was expected that the 1000/100,000 sample would be read as 1% methylated (1000 copies of methylated and 100,000 copies of unmethylated). That some samples were slightly higher (*e.g.*, 4.8% for *HIN1* and 2.8% for *TWIST*) probably reflects difficulty in accurately diluting samples from a starting concentration of 3×10^{10} copies/μl (diluted over 5–10 logs).

To characterize the behavior of the QM-MSP assay below the lower limit of linearity (specifically, whether a 1% M template produces a 1% M assay result as the total quantity of DNA template diminishes), we serially diluted a "1%" standard and tested samples beginning at a template quantity estimated to contain 300 copies of methylated DNA to ~ 29,700 unmethylated copies (300/29,700 per well), using column-purified DNA as a template (Table 3). The lowest quantity tested contained 3 copies of methylated DNA to ~297 copies of unmethylated DNA (3/297). The results for *RASSF1A* and *TWIST* (Table 3) revealed that at ratios of 300/29,700 and 30/2,970 total copies, the

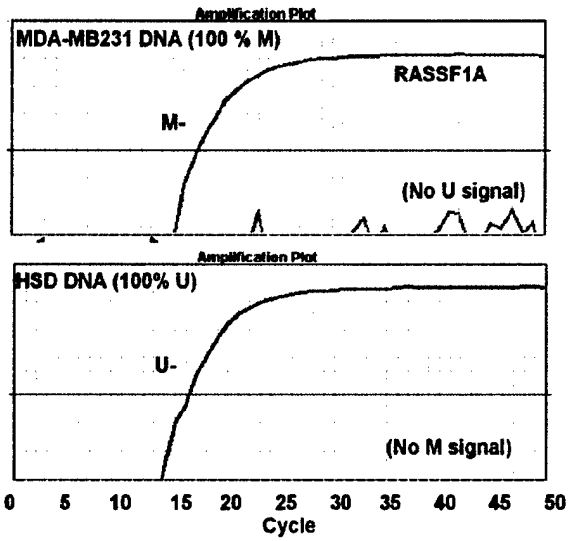


Fig. 3. Specificity of quantitative multiplex methylation-specific PCR. Shown are amplification plots of multiplexed human sperm DNA (*HSD*) or MDA-MB231 template DNA tested with unmethylated (*U*) and methylated (*M*) primers for *RASSF1A*. The unmethylated and methylated reactions were 100% specific because U primers did not cross-react with MDA-MB231 DNA (100% methylated) and M primers did not cross-react with human sperm DNA (100% unmethylated).

assay result (% M) was ~1% for each. Methylation was still detectable at the lowest ratio template quantity tested, at 3 copies of methylated DNA, consistent with the previous experiment (1 copy of methylated DNA was detected in 100,000 copies unmethylated DNA). We found a bias toward underreporting of the % M below 30 copies of methylated DNA, probably reflecting the relative lack of efficiency of the methylated reaction compared with the unmethylated reaction that contained nearly 100-fold more copies of the gene (Table 3). This result was predicted because linearity is generally known to be lost below 100 copies of DNA template in real-time PCR.

Genomic DNA is a more challenging template than PCR-amplified DNA because breakage of genomic DNA is known to occur in the process of sodium bisulfite conversion. To evaluate the sensitivity of the QM-MSP method for detecting methylated alleles when genomic DNA was used, we mixed ~40 pg of methylated DNA (~13 copies derived from 231 cell DNA) with 600–60000 pg of unmethylated genomic DNA (~200–20,000 copies of HSD), using the conversion estimate of 3 pg/copy of genomic DNA. Our data showed that 40 pg of methylated *RASSF1A* genomic DNA was easily detected even in the presence of a 1500-fold excess of unmethylated DNA (Fig. 4). F4

To ensure specific amplification of sodium bisulfite-modified DNA (but not nonconverted DNA; Ref. 41), each primer set (forward, reverse, and probe) covered 7–12 CpG dinucleotides pairs when used together and also covered numerous independent (presumably unmethylated) cytosines. Controls were included during the development of QM-MSP to ensure that no reactivity with nonconverted or sodium bisulfite-treated carrier (salmon sperm) DNA was detectable by QM-MSP (data not shown). Lastly, in each QM-MSP reaction, 100% unmethylated (HSD) and 100% methylated (231) sodium bisulfite-modified DNAs were analyzed as controls, and no background signals were detectable with use of real-time primers.

Comparison of QM-MSP with Direct Q-MSP. The QM-MSP method is sensitive and specific (Table 3; Fig. 3). There are two basic differences between most conventional Q-MSP methods and QM-MSP: (*a*) QM-MSP includes an additional PCR step (multiplex), which could lead to greater sensitivity but lower specificity than Q-MSP; and (*b*) in contrast to the standard use of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or *ACTB* reference DNA, QM-MSP uses the sum of methylated and unmethylated DNA (U + M) of the same gene for determining total gene DNA present in the sample. The use of the (U + M) formula ignores the possible contribution to total DNA from partially methylated DNA and thus could potentially overestimate the percentage of methylation in each sample. We tested the impact of these differences experimentally.

QM-MSP *versus* Direct Q-MSP Using (U + M) as Total DNA. It is possible that performing a two-step multiplex PCR method could yield results that differed from those obtained with a direct one-step PCR method because of the addition of the multiplex step. We performed QM-MSP and direct Q-MSP assays on a panel of five tumor DNAs and calculated the percentage of methylation by the (U + M) method to estimate total DNA. With few exceptions, there was excellent concordance between the percentage of methylation values obtained for the *RASSF1A*, *TWIST*, *HIN1*, or *Cyclin D2* genes (Table 4). The QM-MSP readout was much more robust (as a result of the preamplification of DNA), usually appearing around cycles 12–25 compared with the readout by Q-MSP, in which the C_T signal appeared around cycles 27–37 (data not shown). T4

Comparison of Percentage Methylation Using Actin (*ACTB*) *versus* (U + M) Target Gene DNA to Approximate Total DNA. We first addressed the question of whether the *ACTB* reference method and (U + M) methods yielded similar data in the setting of Q-MSP, as described in the "Materials and Methods." We found that, with few exceptions, there was excellent concordance between the % M values when we used either of the *ACTB* formulas or the (U + M) formula to estimate total DNA in Q-MSP (data not shown).

We next tested the same concept in the QM-MSP setting. For the same DNA samples, discordance was observed for % M values between (U + M) or *ACTB* formulas (data not shown). The rate of amplification of *ACTB* compared with those of the other genes in the mixture was not predictable in the multiplex reaction. For example, values obtained with *ACTB* as the reference gene (as described in the "Materials and Methods") showed concordance for some genes, very high values for others, and lower methylation values in ~50% of the samples (data not shown). On the other hand, with the (U + M) method, QM-MSP values were reproducible across quadruplicate assays. For example, different tumors gave % M values for *RASSF1A* of 42.6 ± 3.5, 42 ± 6.9, 71 ± 1.5, 13 ± 6.0, 27 ± 7.5, 81 ± 1.5, and

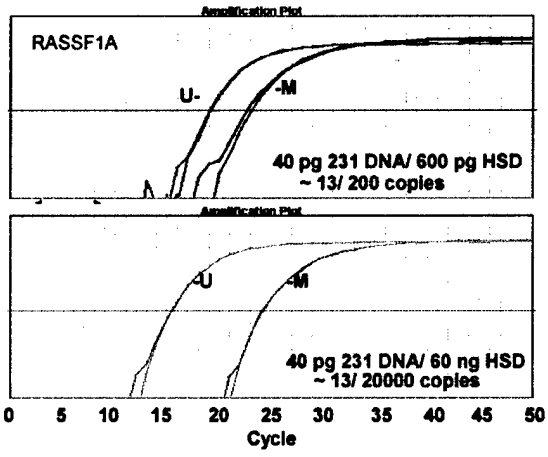


Fig. 4. Sensitive detection of hypermethylated genomic *RASSF1A* DNA. Genomic human sperm DNA (*HSD*) and MDA-MB231 (*231*) DNA were mixed as indicated before sodium bisulfite treatment (*top panel*, 600 pg of HSD and 40 pg of 231 DNA; *bottom panel*, 60 ng of HSD and 40 pg of 231 DNA). Quantitative multiplex methylation-specific PCR was performed. Shown are the amplification plots of each mixture of template. The plots show that 40 pg of genomic DNA is easily detectable even in the presence of a 1500-fold excess of unmethylated DNA.

Table 4 *Comparison between Q-MSP[a] and QM-MSP*

The percentage of methylation (% *M*) was calculated as the number of copies of methylated DNA divided by the number of copies of unmethylated + methylated DNA × 100, using absolute quantitation. The positive control for unmethylated DNA was HSD, and for methylated DNA was 231.

	% M	
	Q-MSP	QM-MSP
RASSF1A		
1	95	99
2	80	81
3	39	41
4	12	21
5	28	34
HSD	0	0
231	100	100
Water	0	0
TWIST		
1	96	70
2	0	0
3	54	26
4	1	3
5	0	5
HSD	0	0
231	100	100
Water	0	0
HIN1		
1	95	82
2	81	74
3	82	48
4	41	16
5	47	43
HSD	0	0
231	100	100
Water	0	0
Cyclin D2		
1	47	25
2	0	0
3	1	0
4	19	22
5	2	0
HSD	0	0
231	100	100
Water	0	0

[a] Q-MSP, quantitative methylation-specific PCR; QM-MSP, quantitative multiplexed methylation-specific PCR; HSD, human sperm DNA.

41 ± 2.0%, whereas DNA samples that were negative for methylation yielded % M values of 0, 0, 0.4 ± 1.0, and 1.5 ± 0.5%. In QM-MSP, the sum of the unmethylated and methylated alleles of the same gene appear to serve as a reliable internal control for integrity, copy number, and method efficiency. The (U + M) formula was therefore used for the rest of the study.

Quantitation of Methylation in Invasive Breast Carcinoma and Comparison with Normal Breast Tissue. We analyzed test sets of DNA from 18 normal mammoplasty and 21 tumors specimens by QM-MSP for gene promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 5; Fig. 5). For *RASSF1A* alone, the normal breast test set was further expanded to 28 samples based on a previous report of a higher incidence of hypermethylation in benign breast tissue (15). Occasionally, PCR amplification failed for some genes within a test sample, presumably because of the fragile nature of archival DNA. *RASSF1A* hypermethylation ranged from 0 to 71% (mean, 18.5%) in carcinoma and from 0 to 56% (mean, 2.6%) in normal tissues ($P = 0.0001$), *TWIST* hypermethylation ranged from 0 to 72% (mean, 21.1%) in carcinomas and from 0 to 1.6% (mean, 0.11%) in normal tissues ($P = 0.0001$), *Cyclin D2* hypermethylation ranged from 0 to 44.5% (mean, 5.0%) in carcinomas and from 0 to 0.2% (mean, 0.02%) in normal tissues ($P = 0.02$), and *HIN1* hypermethylation ranged from 0 to 82.2% (mean, 24.5%) in carcinomas and from 0 to 18% (mean, 2.3%) in normal tissues ($P = 0.003$). When we used the Mann–Whitney test on untransformed data, the differences in the medians were highly significant for all genes tested: *RASSF1A* ($P = 0.0001$), *TWIST* ($P = 0.001$), *Cyclin D2* ($P = 0.0009$), and *HIN1* ($P = 0.003$). We also analyzed normal leukocyte DNA and found that methylation in these samples derived from the buffy coat was extremely low or undetectable ($n = 25$): median leukocyte methylation was 0% for *RASSF1A*, 0.06% for *TWIST*, 0% for *Cyclin D2*, 0.005% for *HIN1*, and 0.25% for *RARβ*. Because DNA from mammoplasty specimens and leukocytes is largely unmethylated relative to tumor samples, it is likely that the methylation signals observed in the tumors (Fig. 5) are derived largely from the carcinoma cells rather than normal ducts, stroma, and/or infiltrating leukocytes.

We chose to establish a cutoff (% M) for each gene at approximately the 10th percentile of the population, such that ~90% of normal breast tissues would be at or below the cutoff (we allowed 85–90% for *HIN1*; see above). Using cutoffs of 2% M for *RASSF1A* and *HIN1*, 0.5% M for *TWIST*, and 0.2% M for *Cyclin D2* in normal tissues, we considered values above the cutoffs "positive" for hypermethylation. Among carcinomas, 68% were positive for *RASSF1A*, 67% for *TWIST*, 57% for *Cyclin D2*, and 57% for *HIN1*. By comparison, 7–14% of normal mammoplasty samples were positive for *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*. Some samples had low-level methylation that was below the cutoff. Using these cutoffs, we observed a significant difference in the incidence of positivity between carcinoma and normal tissues (*RASSF1A*, $P < 0.00002$; *TWIST*, $P < 0.0002$; *Cyclin D2*, $P < 0.002$; and *HIN1*, $P < 0.02$, Fisher's exact).

Cumulative Gene Promoter Hypermethylation Scores in Primary Breast Cancer. To calculate the total amount of gene promoter hypermethylation as determined by QM-MSP, we used the sum of all

Table 5 *Quantitative multiplexed methylation-specific PCR analysis of normal breast and breast cancer DNA*

	Methylation (%)					
	Range	Median	Mean ± SE	Lower 95% confidence limit[a]	Upper 95% confidence limit	Positive for methylation,[b] n (%)
Normal breast						
RASSF1A	0–56	0[c]	2.6 ± 2.0	(0)	6.7	2/28 (7)
TWIST	0–1.6	0[d]	0.11 ± 0.09	(0)	0.29	1/18 (6)
HIN1	0–18	0[e]	2.3 ± 1.5	(0)	5.5	2/14 (14)
Cyclin D2	0–0.2	0[f]	0.019 ± 0.014	(0)	0.48	1/16 (7)
Breast carcinoma						
RASSF1A	0–71	7.0[c]	18.5 ± 4.7	8.7	28.2	13/19 (68)
TWIST	0–72	5.0[d]	21.1 ± 5.5	9.6	32.6	14/21 (67)
HIN1	0–82	9.9[e]	24.5 ± 6.1	11.8	37.3	12/21 (57)
Cyclin D2	0–44	0.26[f]	5.0 ± 2.5	(0)	10.3	12/21 (57)

[a] Values in parentheses indicate ●●●.
[b] Based on percentage of methylation cutoffs of 2% for *RASSF1A* and *HIN1*, 0.5% for *TWIST*, and 0.2% for *Cyclin D2*.
[c] $P = 0.0001$ for *RASSF1A*.
[d] $P = 0.001$ for *TWIST*.
[e] $P = 0.003$ for *HIN1*.
[f] $P = 0.0009$ for *Cyclin D2*.

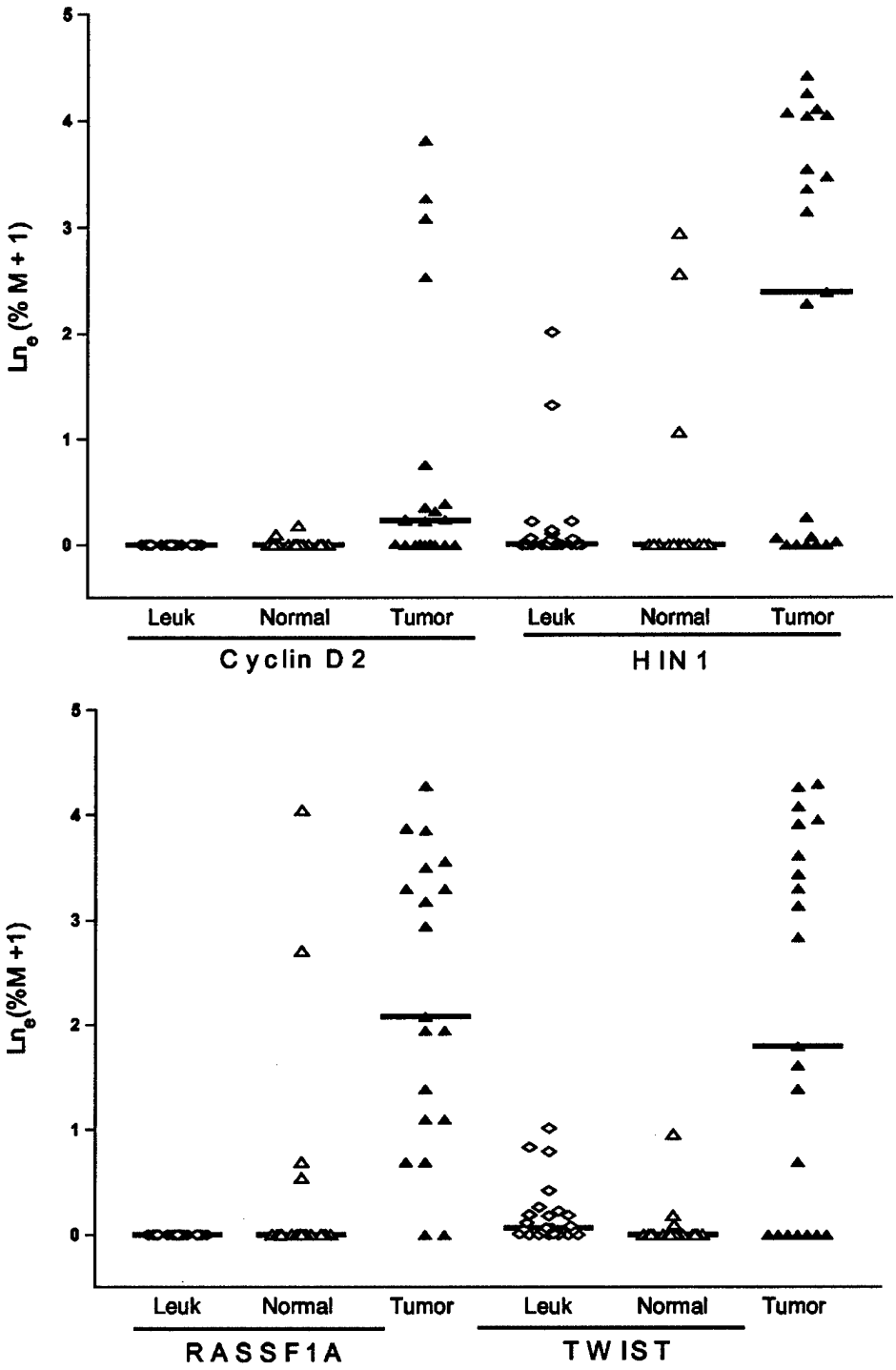


Fig. 5. Comparison of gene promoter hypermethylation in normal and malignant breast tissues. Quantitative multiplex methylation-specific PCR was performed on DNA from formalin-fixed, paraffin-embedded normal tissues, derived from patients undergoing reduction mammoplasty, for quantitation of *RASSF1A* (n = 28), *TWIST* (n = 18), *Cyclin D2* (n = 16), and *HIN1* (n = 14) gene promoter hypermethylation. In parallel, DNA derived from individuals with invasive ductal carcinoma was tested for *RASSF1A* (n = 19), *TWIST* (n = 21), *Cyclin D2* (n = 21), and *HIN1* (n = 21). Displayed is the Ln_e(% M + 1). The median of each group is indicated by a *horizontal bar*. Percentage methylation values for normal and carcinoma DNA were significantly different for all genes. Leukocyte (*Leuk*; n = 25) DNA derived from normal individuals showed low to no levels of methylation.

% M within the panel of genes to provide an overall cumulative score for each sample. In Fig. 6*A*, this is represented graphically relative to 231 DNA, which is 100% methylated for *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*; therefore, this control DNA had a relative score of 400. The cumulative methylation profiles of 9 normal mammoplasty samples were compared with those of 19 invasive carcinomas (Table 6; Fig. 6) in a subgroup of our test set in which results for all four markers were available. Normal tissues ranged from 0 to 18 units, and carcinomas ranged from 1 to 248 units. Among the nine normal tissues tested for four genes (36 values) the mean cumulative score was 2.61 ± 2.05 (median = 0; Fig. 6*B*). Among the 19 carcinomas tested for four genes (76 values), the mean cumulative score was 72.8 ± 15.03 units (median = 74) out of a possible 400 units (see above; Table 6; Fig. 6). The difference in log-transformed means between normal and malignant breast tissue was highly significant (P = 0.0001, unpaired t test with Welch's correction).

We explored the use of 4.7 units as a cumulative score cutoff value. This was based on the individual gene cutoffs (2% each for *RASSF1A* and *HIN1*, 0.5% for *TWIST*, and 0.2% for *Cyclin D2* = 4.7%). When we used this cutoff, 84% (16 of 19) of carcinoma samples were positive. Although 3 of 19 carcinomas fell below the cutoff, all of the "negative" carcinomas were methylated at low levels for one or more

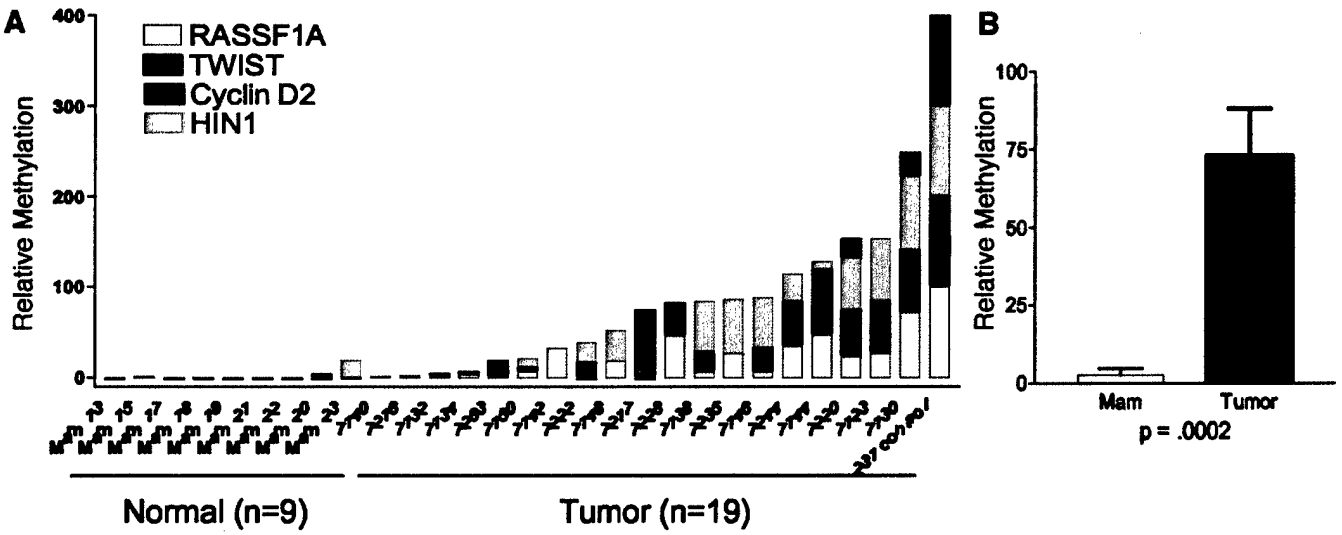


Fig. 6. Cumulative promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* in normal and malignant breast tissues. *A*, scores of cumulative methylation in normal mammoplasty and carcinoma. In a subgroup of samples from Fig. 5 for which results were available for all four genes in the panel, normal (*Mam*; $n = 9$) and carcinoma (*Tumor*; $n = 19$) samples were scored for cumulative methylation by adding the percentage of methylation for all four genes within each sample. A maximum of 400 relative methylation units was possible [*e.g.*, MDA-MB231 (*231 control*) DNA is 100% methylated for each of the four genes]. Results are plotted as *stacked bar graphs*. The *bar height* reflects total cumulative methylation, whereas the *segments* corresponding to the relative amounts of methylation of each gene are indicated. *B*, mean cumulative methylation in normal mammoplasty (*Mam*) *versus* carcinoma (*Tumor*). Plotted is the mean (± SE; *bars*) amount of cumulative methylation.

genes in this panel (Fig. 6*A*). Among normal samples, 89% (8 of 9) were negative. Thus, in this group of 28 samples (9 normal and 19 carcinoma), the sensitivity for detection of carcinoma was 0.84 and specificity was 0.89; the overall accuracy was 0.86 (24 of 28).

Comparison of Paired Carcinoma and Adjacent Normal Breast Epithelium. In an independent experiment, we examined six pairs of carcinoma and adjacent tissue from the surgical margins that were histologically normal to determine the cumulative amount of gene promoter hypermethylation in *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 6; Fig. 7). The cumulative methylation ranged from 2 to 29 units within adjacent normal tissues and from 5 to 258 units within carcinoma tissues, out of a possible 400 units (Fig. 7*B*). When we used the cutoff established for cumulative normal in mammoplasty samples (≤4.7 units; see above), all six carcinomas were positive. The adjacent "normal" tissues were also positive in four of six individuals. Although the cumulative methylation levels within carcinoma-adjacent, histopathologically normal tissues were significantly lower than in the nearby carcinoma ($P = 0.03$, Mann–Whitney), they had a significantly higher levels of methylation than normal mammoplasty samples ($P = 0.01$, Mann-Whitney; Table 6; Fig. 7*B*).

Detection of Multigene Promoter Hypermethylation in Breast Ductal Cells. To test the applicability of this method to investigate small clinical samples, we performed QM-MSP to assess *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* gene promoter hypermethylation in ductal cells derived from lavage of high-risk women ($n = 7$) or cells in the irrigation fluid obtained during breast endoscopy in four patients with biopsy-proven cancer, before resection of the lesion. The total number of epithelial cells present in the samples was estimated to be 50–1000 cells. The level of gene promoter methylation was quantitated for each sample, and the cumulative promoter methylation profile was established. Six of seven samples from women with a high risk of developing breast cancer had no detectable hypermethylation in the five genes tested, whereas one of seven displayed low-level methylation in *RASSF1A*. Cytological diagnosis of all of the specimens in this group was "benign." Of interest, cells obtained during endoscopy from the two women with invasive carcinoma had high-level multigene promoter hypermethylation (samples 10 and 11 in Table 7 and Fig. 8). The samples from the two women with ductal carcinoma *in situ* displayed benign cytology and lacked detectable promoter hypermethylation (samples 8 and 9).

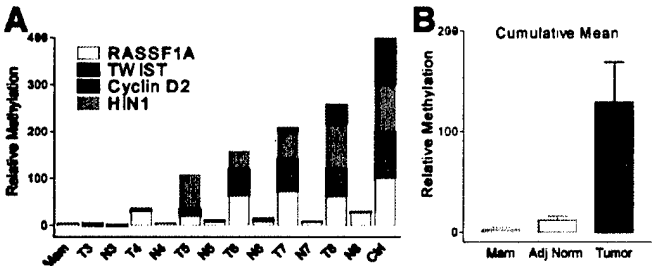
DISCUSSION

Cytological analysis of breast ductal cells could aid in assessing risk and in the early detection of breast cancer, particularly in young women. To provide a robust and objective method to analyze these

Table 6 *Cumulative promoter hypermethylation in normal, adjacent "normal" and malignant breast tissues*

Tissue	Methylation (units)[a] Range	Median	Mean ± SE	Lower 95% confidence limit[b]	Upper 95% confidence limit	Positive for methylation,[c] n (%)	n
Normal mammoplasty and malignant breast tissues							
Normal	0–18	0[d]	2.61 ± 2.05[e]	(0)	7.35	1/9 (11)	9
Carcinoma	0–248	74[d]	72.8 ± 15.03[e]	41.3	104.4	16/19 (84)	19
Paired malignant breast tissues and their adjacent normal breast tissues							
Adjacent normal	2–29	9[f]	11.7 ± 4.07	1.2	22.1	4/6 (67)	6
Carcinoma	5–258	133[f]	129.2 ± 39.9	26.5	231.8	6/6 (100)	6

[a] Relative units of methylation is the sum of percentage methylation for each of four genes in the panel.
[b] Value in parentheses indicates ●●●.
[c] Based on a cutoff of ≤4.7 units.
[d] $P = 0.0001$.
[e] $P = 0.0002$.
[f] $P = 0.03$.



Gene	% Methylation 3 T	3 N	4 T	4 N	5 T	5 N	6 T	6 N	7 T	7 N	8 T	8 N	Ctrl
RASSF1A	1	0	30	4	19	8	63	8	72	7	61	27	100
TWIST	3	1	2	0	17	2	58	5	69	1	61	2	100
Cyc D2	1	1	0	0	0	0	0	0	9	0	44	0	100
HIN1	0	0	6	0	72	0	37	4	58	0	92	0	100
Total	5	2	38	4	108	10	158	17	208	8	258	29	400

Fig. 7. Cumulative promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* in adjacent normal and malignant breast tissues. *A*, cumulative methylation in adjacent normal and carcinoma tissues. In an independent experiment, paired samples (n = 6) of adjacent normal and carcinoma tissue were quantitated by quantitative multiplex methylation-specific PCR, and the extent of cumulative methylation of the gene panel was determined, as described in the legend for Fig. 6. Shown are results of each sample as well as positive control MDA-MB231 DNA (*Control*; 400 units). For purposes of comparison, also shown at the *far left* is the average cumulative methylation of normal mammoplasty (n = 9) samples (*Mam*) from Fig. 6*B*. Raw scores are shown in the *table*. *B*, mean cumulative methylation in adjacent normal (*Adj Norm*) *versus* carcinoma (*Tumor*) compared with normal mammoplasty (*Mam*). Differences between normal mammoplasty samples (median = 0) and adjacent normal tissue samples (median = 9 units) were significant (P = 0.01, Mann–Whitney test). Plotted is the mean (± SE; *bars*) amount of cumulative methylation found above in adjacent normal tissues (mean, 11.7 ± 4.07) and the nearby carcinoma (mean, 129 ± 39.9) compared with normal mammoplasty (mean, 2.61 ± 2.05; as shown in 6*B*). Actual percentage of methylation values are listed in the *table* for tumor (*T*), adjacent normal (*N*), and MDA-MB231 positive control (*Ctrl*).

cells, in this report we describe a technique called QM-MSP. We show that QM-MSP evaluates the level and incidence of promoter hypermethylation of several genes in samples where DNA is limiting. Scoring the cumulative methylation of these genes within a sample provided a high level of sensitivity and specificity of detection of cancer. We tested this method in clinical settings where samples yield very limited amounts of DNA, such as those from ductal lavage and ductal irrigation fluid collected during endoscopy. In the process, we more clearly defined the extent of gene promoter hypermethylation in normal breast cells and showed the feasibility for adapting this method to clinical testing. Although we applied this technique to breast tissues, it can be used to evaluate gene promoter hypermethylation in a wide variety of tissues.

The expression of >40 genes is reportedly lost in breast cancer because of promoter hypermethylation (4). Recent work in our laboratory and by others has shown that some of the genes most frequently hypermethylated (30–90%) in breast carcinomas, but not in normal breast epithelium or circulating blood cells, are *Cyclin D2* (14, 15), *RARB* (16–18), *TWIST* (19), *RASSF1A* (15, 20, 21), and *HIN1* (22). In a study of 103 cases of breast cancer we recently reported that 100% of cases of invasive carcinoma and 95% cases of ductal carcinoma *in situ* were hypermethylated for one or more gene promoters in a panel of these five genes (23). In fact, the vast majority of carcinomas (80%) were hypermethylated for two or more of these five genes. From our work we developed support for the concept that profiling the cumulative methylation of multiple genes would serve to better distinguish

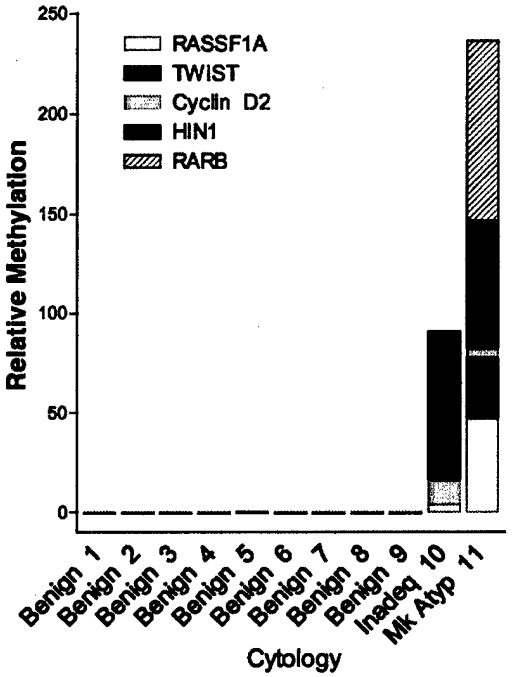


Fig. 8. Cumulative promoter hypermethylation in ductal fluid. *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* genes were coamplified, and promoter hypermethylation levels were quantitated by quantitative multiplex methylation-specific PCR in ductal cells derived from lavage of high-risk women (n = 7) or from cells present in the irrigation fluid obtained during breast endoscopy in patients with biopsy-proven cancer (n = 4), before resection of the lesion. *Inadeq*, inadequate sample available; *Mk Atyp*, markedly atypical.

AQ: L

Table 7 *Quantitative multiplexed methylation-specific PCR analysis of ductal breast cells*

ID[a]	Methylation (%) *RASSF1A*	*TWIST*	*Cyclin D2*	*HIN1*	*RARB*	Cytology	Mammography	Histopathology
Ductal lavage cells from high-risk women[b]								
1	0	0	0	0	0	Benign	Normal	
2	0	0	0	0	0	Benign	Normal	
3	0	0	0	0	0	Benign	Normal	
4	0	0	0	0	0	Benign	Normal	
5	0	0	0	0	0	Benign	Normal	
6	0	0	0	0	0	Benign	Normal	
7	0.4	0	0	0	0	Benign	Normal	
Ductal lavage cells from women with carcinoma[c]								
8	0	0	0	0	0	Benign		DCIS
9	0	0	0	0	0	Benign		DCIS
10	4	0	12	75	0	Inadequate		Invasive carcinoma
11	47	31	4	65	89	Markedly atypical		Invasive carcinoma

[a] ID, identification; DCIS, ductal carcinoma *in situ*.

[b] High-risk women with no mammographic abnormality.

[c] Ductal cells retrieved from irrigation fluid during endoscopy of women with biopsy-proven cancer. Histopathological diagnosis of the resected lesion from these breasts showed ductal carcinoma in situ or invasive ductal carcinoma.

benign from malignant tissues and would provide a more powerful approach than characterizing the status of only one gene marker.

Studies in our laboratory have also demonstrated the feasibility of assessing gene promoter hypermethylation in ductal lavage samples (19). We found *TWIST*, *Cyclin D2*, or *RARB* gene promoter hypermethylation in cells derived from patients with ductal carcinoma. Because the ductal cell samples most often contained 50–1000 epithelial cells, assessment of the status of more than three genes in replicate assays could be extremely difficult. There is clearly a need for new strategies to better evaluate methylation in samples where DNA is limited (*e.g.*, ductal lavage, plasma, fine-needle or core biopsy, or nipple aspiration fluid).

The QM-MSP method combines the principles of M-MSP (30, 31, 33) with quantitative real-time MSP (15, 34–39). We have shown that this method can detect as few as 1–10 methylated copies of DNA in a mixture of ~100,000 copies of unmethylated DNA (Table 3; Fig. 3) and 40 pg of methylated genomic DNA in up to 1500-fold excess unmethylated DNA (Fig. 4). This compares favorably with Q-MSP, which has a sensitivity of 1:10,000 (36), and conventional MSP, which has a sensitivity of 1:1000 (30). Reactions were specific: no cross-reactivity was observed between methylated and unmethylated primers even in mixtures containing a $>10^5$-fold excess of one or the other DNA (Fig. 3).

Most often the real-time PCR technology used for absolute quantification of DNA uses β-actin (*ACTB*) or *GAPDH* as reference DNA (34, 36, 37). We have demonstrated that by assessing the levels of unmethylated and methylated product for each gene, it is possible to quantitate the percentage of methylated gene product. Lo *et al.* (35) AQ: G and Wong *et al.* (42) used a similar approach for Q-MSP, although they also considered the contribution of any unconverted bisulfite-treated DNA. However, two potential pitfalls of the QM-MSP method needed to be addressed. One matter of concern is how much bias is introduced into the estimation of gene methylation by the addition of the multiplex reaction to the Q-MSP procedure, as described here. A second concern is that QM-MSP does not take into account the existence of variable fractions of partially methylated DNA in the tissue samples. Therefore, samples could appear to contain higher levels of methylated DNA in the test genes than are present.

By testing the first question experimentally, we showed that, with few exceptions, there was excellent concordance between QM-MSP and Q-MSP for all four genes when we used (U + M) as the measure of total gene DNA (Table 4). Because QM-MSP and Q-MSP give essentially the same readout, significant bias is not likely in QM-MSP. To address the second question, using direct Q-MSP we calculated methylation as done with *ACTB*-based Q-MSP by Trinh *et al.* (37) and as % M by (U + M) (see the "Results"). There was concordance in percentage of methylation calculated by the two *ACTB*-based formulas and the (U + M) formula. Such concordance would be unlikely if partially methylated DNA formed a substantial component of the DNA. In contrast, in the two-step QM-MSP assay, we observed that *ACTB* does not perform predictably. The reasons could be as follows: In the first step, that of multiplex PCR amplification, the efficiency of amplification of each of the genes was not identical, however well optimized. In some samples, *ACTB* did not seem to amplify as well as some of the genes in the mixture. Because the strength of the second Q-PCR reaction depends on the efficiency of the first, differences are magnified in the second reaction. Thus, for QM-MSP we decided to calculate percentage of methylation using the (U + M) formula.

In addition, in QM-MSP, a gene controls for itself. In this assay, the sum of unmethylated and the methylated alleles of the same gene serve as a reliable internal control for integrity, copy number, and method efficiency. Among these, copy number is an important consideration because allelic losses and amplifications can vary among different areas of the genome and between samples. For example, for *RASSF1A* two simultaneous methods of gene inactivation have been observed: loss of one allele and methylation of the other (43). This has also been reported for *FHIT* (44), *APC*, and *CDH1* (13). Wang *et al.* (45) reported that nearly every breast tumor has an individual pattern of allelic imbalance or loss of heterozygosity at multiple loci, which constitutes its "fingerprint."

The QM-MSP technique is applicable to frozen or archival paraffin-embedded clinical tissues (Figs. 5–7) as well as to ductal lavage material (Fig. 8). In a study of 14–28 tissue samples/group, we observed significant differences in the level of promoter hypermethylation between normal and carcinoma samples for each of four genes, based on comparison of mean and median normal values (Fig. 5; Table 5). QM-MSP enabled definition of the normal range for the percentage of methylation in the genes in normal breast tissue (Table 5; Fig. 5). Techniques that give higher sensitivity usually also give higher "background," picking up signals that are missed by other methods. This was also observed in our present study, in which we found a higher incidence of methylation in normal mammoplasty than we did previously (23) using gel-based nonquantitative MSP. Nevertheless, with QM-MSP, the median for normal tissues was 0% M for all genes. By setting an upper threshold for normal, we acknowledge the occasional low-level methylation that occurs in some normal tissues and set criteria that define "positive" in carcinoma. By determining that peripheral blood cells contain little or no methylation of the genes tested and that normal breast tissue, which is rich in stroma, is for the most part negative, we were able to deuce that the methylation signal is derived largely from the epithelial cells (Fig. 5). The incidence of positivity among carcinomas was 68% for *RASSF1A*, 67% for *TWIST*, 57% for *Cyclin D2*, and 57% for *HIN1* (Table 5) according to this stringent criterion. However, all carcinomas showed some degree of methylation of one or more of the genes in our panel.

Studies of cumulative multigene promoter hypermethylation revealed striking differences between normal and malignant tissues (Fig. 6; Table 6). There was a highly significant ($P = 0.0002$) difference between levels of cumulative gene promoter hypermethylation in normal tissues compared with malignant tissues. Cumulative methylation profiling of four genes was able to detect 84% of carcinomas, whereas single-gene analyses yielded positive results in only 57–68% cases, depending on the gene analyzed (Figs. 5 and 6). To our knowledge, this is the first study to describe quantitation of cumulative methylation and to show its importance in distinguishing between normal and carcinoma tissues.

Molecular alterations in histologically normal-appearing breast tissue adjacent to carcinomas have been reported previously (46–48). Promoter hypermethylation analysis of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 6; Fig. 7) in six pairs of carcinomas and histologically normal adjacent tissues showed that all six carcinomas were positive. Four of six adjacent normal tissues were also positive, although the levels were considerably lower than in the carcinoma ($P = 0.01$, Mann–Whitney test). More detailed studies are needed to determine whether methylation in histologically normal tissue adjacent to carcinoma represents a "field effect" presaging cancer or are normal, age-related changes. That this may represent a field effect is suggested by our observation that in 25 samples of normal breast tissue, including those reported here, we have not observed a correlation between age and methylation in these five genes.[6] AQ: H

That it is possible to apply the QM-MSP successfully to samples with little cellularity was demonstrated by our pilot study with ductal cells retrieved by lavage or endoscopy (Fig. 8; Table 7; Ref. 19). In

[6] Our unpublished data.

the seven DL samples from high-risk, but mammographically normal breasts, no promoter hypermethylation was detectable. In contrast, ductal lavage samples obtained during endoscopy of two women with invasive carcinoma had high-level multigene promoter hypermethylation, consistent with the histological diagnosis of the resected tissue. Interestingly, samples from the two women with ductal carcinoma *in situ* demonstrated benign cytology and lacked detectable promoter hypermethylation (samples 8 and 9). The sample size is small, and an ongoing clinical trial collecting ductal cells from both diseased and uninvolved breasts of cancer patients will allow us to address the utility of QM-MSP in greater detail.

With the QM-MSP approach, it is possible to put together several gene panels consisting of scores of genes that are designed for early detection or to provide intermediate markers or endpoints for clinical protocols. For example, when retinoids or demethylating agents are being used as chemopreventive agents, a panel can be designed to query pathway-specific genes for their use as intermediate markers in clinical trials (26). Furthermore, the QM-MSP method is applicable to all types of cancer and evaluation of methylated tumor DNA in other small clinical samples, such as prostatic fluid, bile duct washings, and fine-needle aspirates.

In summary, we describe a method that assesses the gene promoter hypermethylation status of multiple genes, using only picograms of DNA. We demonstrate the advantages of a cumulative score of promoter hypermethylation among multiple genes and how this approach may better distinguish normal/benign from malignant tissues. With QM-MSP it is possible to objectively define the range of normal/abnormal gene promoter hypermethylation in a manner that could translate to a larger clinical setting. Further studies should examine cumulative hypermethylation in benign conditions and as a predictor of breast cancer risk.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Saraswati Sukumar, Ph.D.	Professor of Oncology/Pathology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Nagpur University, Nagpur, India	M.S.	1969	Biochemistry
Atomic Energy Commission at Cancer Institute, Madras, India	Research Fellow	1971-76	Microbiology
Nagpur University, Nagpur, India	Ph.D.	1977	Biochemistry
National Cancer Institute, NIH, Bethesda, MD	Postdoc Fellow	1978-83	Immunology, Molecular Biology

A. Position and Honors

1976-78 Assistant Research Officer, in charge of Tumor Biology Section, Indian Council of Research in Indian Medicine, Madras, India.

1978-83 Visiting Associate, Laboratory of Immunobiology, National Cancer Institute, Bethesda, MD (Section Chief, B.Zbar)

1983-88 Scientist Associate, NCI and Bionetics Research Inc., NCI/Frederick Cancer Research Facility, Frederick, MD (Section Chief, Mariano Barbacid)
Senior Research Associate, Dulbecco Laboratory, The Salk Institute for Biological Studies LaJolla, CA

1989-94 Assistant Professor, Molecular Biology of Breast Cancer Laboratory, The Salk Institute for Biological Studies, LaJolla, CA

1994-02 Associate Professor of Oncology and Director of Basic Research, Breast Cancer Program, The Johns Hopkins University School of Medicine, Baltimore, MD
Associate Professor of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD

2002- Barbara B. Rubenstein Professor of Oncology and Pathology, Director of Basic Research; Breast Cancer Program, The Johns Hopkins University School of Medicine, Baltimore, MD

Scientific Counselor, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 1991-94 Member of the Board of Governors, International Association for Breast Cancer Research, 1991-94

Member, American Cancer Society Grant Review Committee, 1992-1998

B. Selected peer-reviewed publications (out of 70)

Sukumar S, Notario V, Martin-Zanca D, Barbacid M: Induction of mammary carcinomas in rats by nitroso-methyl-urea involves the malignant activation of the H-*ras*-1-locus by single point mutations. Nature 306:658, 1983.

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